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Review

Plasma non-cholesterol sterols

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Abstract

Increased levels of plasma sterols other than cholesterol can serve as markers for abnormalities in lipid metabolism associated with clinical disease. Premature atherosclerosis and xanthomatosis occur in two rare lipid storage diseases, Cerebrotendinous xanthomatosis (CTX) and sitosterolemia. In CTX, cholestanol is present in all tissues. In sitosterolemia, dietary campesterol and sitosterol accumulate in plasma and red blood cells. Plasma accumulation of oxo-sterols is associated with inhibition of bile acid synthesis and other abnormalities in plasma lipid metabolism. Inhibition of cholesterol biosynthesis is associated with plasma appearance of precursor sterols. The increases in non-cholesterol sterols, while highly significant, represent only minor changes in plasma sterols, which require capillary gas–liquid chromatography and MS for effective detection, identification and quantification.

Keywords: Reviews; Cholesterol; Sterols; Lipids

Contents

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1. Introduction

The measurement of cholesterol precursors and plant sterols as well as their reduction and oxidation products in lipoproteins is useful in the study of hypercholesterolemia, sitosterolemia, and potentially atherosclerosis and heart disease. The major sources of plasma non-cholesterol sterols are due to systemic circulation of biosynthetic precursors of cholesterol, and their metabolic transformation products. The complex mixtures, relatively low concentrations and chemical instability of these steroids present special problems in their isolation, identification and quantification. The present review provides an update of the methodology [1,2] as well as other areas [3] of oxo-sterol research. Since the analytical problems are directly related to the origin of the non-cholesterol sterols, this review has been organized around the major sources of the non-cholesterol steroids.

2. Cholesterol precursors

The cholesterol precursors are measured as an indicator of cholesterol biosynthesis. The rationale for measuring the serum level of these precursors lies in the assumption that these compounds in their unesterified form leak into plasma lipoproteins at a rate proportional to that of their formation in the cholesterol synthetic pathway. Only the unesterified methylsterols in serum are considered to reflect cholesterol synthesis, since their esterification can only take place in the liver by acyl cholesterol acyltransferase (ACAT) [4] and not in plasma by lecithin cholesterol acyltransferase (LCAT) [5]. The metabolism of the plasma or serum methylsterols and lathosterol is further discussed by Kempen et al. [6] and Bjorkhem et al. [7].

In the past, various precursors along the cholesterol synthesis pathway have been proposed for this purpose with special emphasis on free methylsterols [8]. Vuoristo and Miettinen [9] have reported that the serum level of 7-lanosterol (5α-cholest-7-en-3β-ol) in humans decreases upon expansion and increases upon depletion of the bile acid pool, in a more pronounced way than free methylsterols. Kempen et al. [6] have concluded that of the various indicators proposed to monitor whole-body cholesterol synthesis, the lathosterol/cholesterol ratio in serum appears preferable with respect to indicative power and ease of quantification. Each of the precursors of cholesterol synthesis requires its own special method of isolation, identification and quantification. Fig. 1 gives the structures of some of the sterols believed to be involved as intermediates in cholesterol biosynthesis [10].

2.1. Isolation, identification and quantification

Over the years the methodology has improved but certain basic routines have been retained. A representative method for the determination of free lathosterol and the sum of free methylsterols in serum has been described in detail by Kempen et al. [6]. It is based on earlier methods developed by Miettinen and co-workers [5,8,9]. Briefly, epicoprostanol (3 μg) was added as internal standard to 1 ml serum, and total lipids were extracted by the method of Folch et al. [11]. The extract was applied on a 20×20 cm thin-layer silica gel 60 plate (Merck, Darmstadt, Germany), one sample per plate. To separate the various lipid classes, the plate was first developed in hexane–toluene (90:10, v/v), and then in heptane–diethyl ether (55:45, v/v). Lipids were localized by spraying with Rhodamine. The free methylsterol band was scraped between their positions of cholesterol and α-tocopherol reference spots. The free methylsterols were eluted from the silica gel with diethyl ether, and the solvent was evaporated. Quantification by gas–liquid chromatography (GLC) was done as described above for lathosterol, including prior derivatization into trimethylsilyl (TMS)

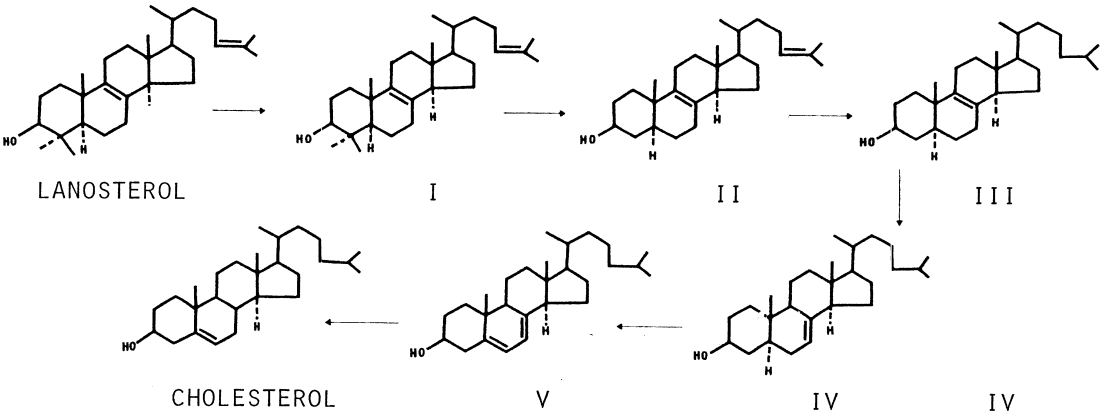

Fig. 1. Probable sterol intermediates in the conversion of lanosterol to cholesterol. I, 4,4-Dimethyl-5α-cholesta-8,24-dien-3β-ol (14α-desmethyllanosterol); II, 5α-cholesta-8,24-dien-3β-ol (zymosterol); III, 5α-cholest-8-en-3β-ol; IV, 5α-cholest-7-en-3β-ol (lathosterol); V, cholesta-5,7-dien-3β-ol (7-dehydrocholesterol).

ethers. Free lanosterol (denoted as peak IV by Miettinen [8]) was identified by Kempen et al. [6] as such in serum extracts by mass spectrometry (MS), and had a relative retention time of 1.81 with respect to epicoprostanol. Other free methylsterols, denoted by Miettinen [8] as peaks I, III, II and V, respectively, had retention times of 1.52, 1.66, 1.77 and 1.92 with respect to epicoprostanol.

Kempen et al. [6] quantified lathosterol by GLC using a Packard 433 chromatograph equipped with a 25 m×0.22 mm I.D. fused-silica capillary column with CP-Sil-5CB as the stationary phase (Chrompack, Middelburg, The Netherlands). Hydrogen was used as the carrier gas; the column was kept at a constant temperature of 235°C. Detection was by flame ionization. Response factors were assumed to be equal among the various steroids. Pure lathosterol (Steraloids, Wilton, NH, USA) had a relative retention time of 1.12 with respect to cholesterol and 0.85 with respect to campesterol. The procedure was found to have an intra-assay relative standard deviation (RSD) of 2% ($n=5$).

Bjorkhem et al. [7] also have reported the measurement of serum levels of free methylsterols and of free and total lathosterol. The precursor sterols were quantified by GLC as described in detail by Miettinen and Koivisto [13]. This procedure involved separation of free and esterified sterols and squalene present in a chloroform–methanol extract of serum lipids by preparative thin-layer chromatography (TLC). For isolation of squalene, hexane–toluene (90:10) was used as solvent. The isolated squalene was quantified by GLC. The plate was redeveloped with heptane–ethyl ether (44:55, v/v) for isolation of free cholesterol and the fractions containing free methylsterols and esterified sterols. After saponification of the ester fraction, the material was chromatographed by TLC for isolation of the esterified cholesterol and methylsterol fractions. After silylation, the resulting four sterol fractions were quantified with GLC on a 35-m long SE-30 capillary column under the following conditions. Starting temperature was 170°C, with a temperature program of 20°C/min up to 265°C. Injector temperature was 300°C and detector temperature was 320°C. The carrier gas (helium) flow was 2.5 ml/min at 14 p.s.i. (1 p.s.i.=6894.76 Pa).

Fig. 2 shows typical GLC patterns of the free cholesterol (A), and the free (B) and esterified (C) methylsterol fractions of human serum [7]. The five major free methylsterols are: lanosterol, $\Delta^{8,24}$-dimethylsterol, Δ^{8}-dimethylsterol, Δ^{8}-methostenol and Δ^{7}-methostenol. Frequently a small amount of dihydrolanosterol was found in front of the Δ^{7}-methostenol peak. The patterns of the free and esterified methylsterol fractions were markedly different. Several other minor unidentified methylsterol peaks occurred in the GLC analyses. Δ^{7}-Lathosterol, Δ^{8}-lathosterol and desmosterol could be identified in the cholesterol fraction. The patterns of the free and

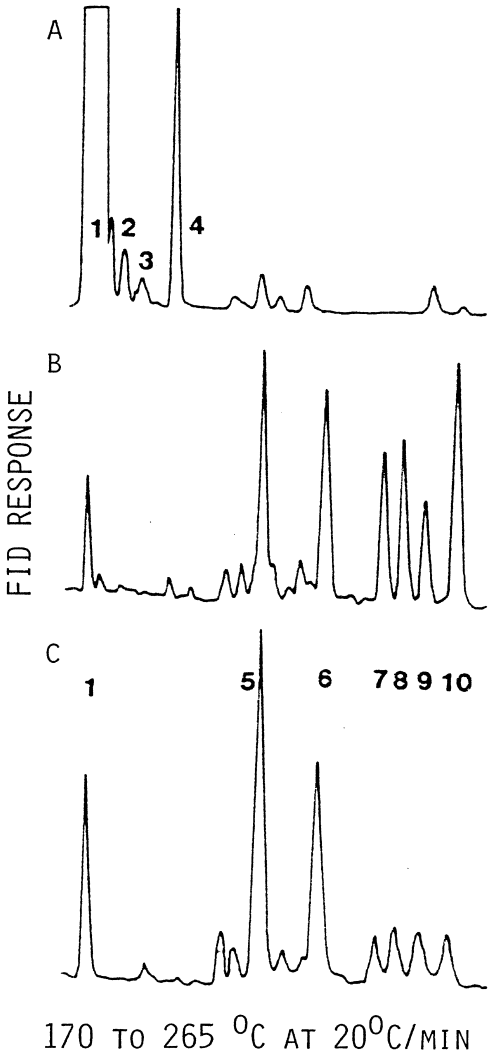

Fig. 2. Gas–liquid chromatographic patterns of free and esterified sterols in human serum. (A) Free cholesterol fraction: 1, cholesterol; 2, Δ^8-lathosterol; 3, desmosterol; 4, Δ^7-lathosterol. (B) Methyl sterols: 1, cholesterol; 5, Δ^8-methostenol; 6, Δ^7-methostenol (including dihydrolanosterol when present); 7, Δ^8-dimethylsterol; 8, lanosterol; 9, unknown; 10, $\Delta^{8,24}$-dimethylsterol. (C) Esterified methylsterol fraction showing predominance of methostenols. Reproduced from Ref. [7] with permission.

esterified cholesterol fractions were quite similar, the esterification percentage of desmosterol being usually higher and that of lathosterol lower than that of cholesterol. Table 1 gives the correlation between the cholesterol precursors in serum and the 3-hydroxy-3 methyl glutaryl (HMG)-coenzyme A (CoA) reductase [7].

The classical method of assessing the rates of whole body cholesterol synthesis in humans, without resorting to use of radioisotopes, comprises measurement of the cholesterol balance, i.e., fecal excretion of cholesterol its plus acidic and neutral metabolites, minus cholesterol intake. Although this method is well established, it has the disadvantage of being laborious and is not suited to detecting changes in cholesterol synthesis occurring within the time scale of less than a few days [6]. Moreover, the method requires that the subjects be in a steady state with regard to cholesterol metabolism. For these reasons, alternative methods have been sought to monitor whole body cholesterol synthesis.

2.2. Lipoprotein distribution

This does not appear to have been determined. Vanhanen et al. [14] have examined the GLC resolution of the cholesterol precursors (squalene, Δ^8-cholestenol, lathosterol, and desmosterol), cholestanol and plant sterols (campesterol and sitosterol, and their saturated forms (campestanol and sitostanol) in the non-saponifiable material of serum. The separations were done using a 50-m long SE-30 capillary column [12].

2.3. Metabolic significance

Vuoristo and Miettinen [9] reported that serum level of 7-lathosterol (5α-cholest-7ene-3β-ol) in humans decreased upon expansion and increased upon depletion of the bile acid pool, in a more pronounced way than free methylsterols. Kempen et al. [6] have claimed that the ratio of serum lathosterol over serum cholesterol strongly correlates with the cholesterol balance under dietary conditions. Moreover, this ratio fell in patients with familial hypercholesterolemia during treatment with a potent HMG-CoA reductase inhibitor. Inhibition of cholesterol biosynthesis was associated with the plasma appearance of the precursor sterols, while inhibition of cholesterol conversion into bile acids was associated with the plasma appearance of the intermediate oxidation products of cholesterol.

Table 1
Correlations between hepatic HMG-CoA reductase activity and absolute concentrations of different precursors to cholesterol in serum ($n=20$)

Cholesterol precursor	Concentration range (μg/100 mg of cholesterol)	Correlation to HMG-CoA reductase (r)	Correlation to total serum cholesterol (mg/dl) (r)
Δ^7-Lathosterol	0–18 487	0.80	0.19
Δ^8-Lathosterol	5–209	0.81	0.10
Desmosterol	42–139	0.63	0.05
Δ^7-Methostenol	7.7–128	0.85	0.02
Δ^8-Methostenol	15.1–106	0.83	0.11
$\Delta^{8,24}$-Dimethylsterol	8.4–108	0.88	0.11
Lanosterol	6.2–83	0.89	0.06
Squalene (μg/dl)	3.9–72	0.20	0.08

The precursor sterols were quantified by gas–liquid chromatography as described in the text. Reproduced from Ref. [7] with permission.

3. Cholesterol oxidation products

Cholesterol shows a particular oxidative behavior because of the double bond and the hydroxy group present in positions 5 and 3 of the cyclic structure, respectively. Cholesterol autooxidation yields mostly two major isomers, one in position 5, the other in position 7, which result from an allylic rearrangement of the 5-hydroperoxide [15]. However, hydroperoxides in positions 4 and 6 are also present in minor amounts. The decomposition of these hydroperoxides can lead to the formation of many compounds, among which oxides in position 7 and the epoxy derivatives are the most abundant. Previous studies have demonstrated that artifactual oxidation of cholesterol may occur during processing and that some cholesterol oxides are formed predominantly as artefacts [16,17]. 7β-Hydroxycholesterol, α-epoxycholosterol, β-epoxycholesterol and cholestane-triol were identified in some samples but at concentrations close to the limit of detection. By addition of [3,4-^{13}C]cholesterol to lipoprotein samples it has been demonstrated that no increase in concentration of 7-ketocholesterol formed by autoxidation may occur during careful sample processing [18]. Fig. 3 gives the chemical structures of some common oxosterols [3].

3.1. Isolation, identification and quantification

Hodis et al. [19] have described the special precautions that are necessary for sample collection and preparation for cholesterol oxide analysis. After

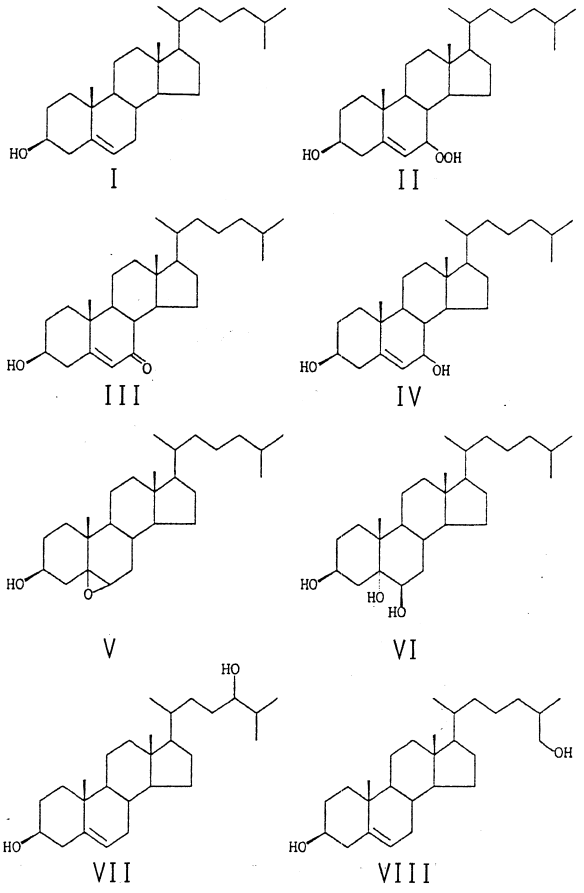

Fig. 3. Chemical structures of some commonly encountered oxosterols. I, Cholesterol; II, 7-hydroperoxycholesterol; III, 7-ketocholesterol; IV, 7-hydroxycholesterol; V, 5,6-epoxycholesterol; VI, cholestan-3β,5α,6β-triol; VII, 25-hydroxycholesterol; VIII, 27-hydroxycholesterol.

a 12 h fast, blood was drawn from the central ear artery of each rabbit at baseline prior to and after 6 weeks of feeding the respective diets. Blood was drawn directly into airless, negatively pressured glass Vacutainer tubes containing 0.07 ml of 15% EDTA (1.5 mg EDTA/ml of blood final volume) and placed immediately on ice. Plasma was separated from the formed elements by centrifugation at 3000 rpm for 20 min while cold and stored under argon in the dark at −70°C until analysis.

Aortae from the aortic valve to the diaphragm were dissected from the animals, immediately washed free of blood with cold phosphate-buffered saline containing 1 mg/ml of EDTA, stripped of all adventitial tissue by sharp dissection, and then immediately frozen at −70°C.

All lipid extraction was conducted under argon utilizing a modified Bligh–Dyer [20] procedure. In brief, 6 ml of chloroform–methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) was added to 1 ml of plasma, mixed vigorously for 5 min, and centrifuged to separate the organic phase, which was collected and saved. The aqueous phase was reextracted as above utilizing three volumes of the solvent, and the organic phases pooled. The lipid were evaporated to dryness under argon and then redissolved in 1 ml of argon-saturated toluene–ethyl acetate (1:1, v/v). As an internal standard, 100 μl of 5α-cholestane (5 mg/ml in toluene) was added to each sample. The same extraction procedure was carried out on the aortic tissue after it was weighed and homogenized under a stream of nitrogen utilizing a Tekmar homogenizer. The samples were then applied to 3 ml solid-phase ''Diol'' extraction columns (VWR Scientific, Cerritos, CA, USA) conditioned with toluene–ethyl acetate and the eluent (the cholesterol and cholesterol oxide fraction) collected under mild vacuum suction. The columns were washed with another 2 ml of toluene–ethyl acetate and 1 ml of chloroform and the eluents pooled.

For the preparation of total sterol extracts from plasma lipoproteins, it is necessary to isolate the lipoprotein fractions in the presence of antioxidants, effective metal-ion chelators and inhibitors of proteolysis. Dyer et al. [18] have described an improved method for the isolation of serum lipoproteins. Separation of serum was achieved by centrifugation of blood at 1000 *g* for 15 min within 1 h of venesection. Serum was mixed immediately with ethylenediaminetetracetic acid (EDTA, disodium salt) as a metal-ion chelator, BHT as antioxidant and phenylmethylsulfonylfluoride (PMSF) as an inhibitor of proteolysis. Final concentrations of each were 0.372, 0.022 and 0.148 g/l, respectively. Serum was stored at −20°C until separated by ultracentrifugation (within 10 days). The separation of lipoproteins was achieved as described previously [21]. Briefly, the density of 2 ml of serum was adjusted to 1.006 g/ml with 8 ml 11.05 g/l NaCl. Specimens were centrifuged at 100 000 *g* for 24 h at 20°C. Very-low-density lipoprotein (VLDL) was recovered in the top 2 ml, the third ml being removed for measurement of refractive index. The density was then adjusted to 1.019 g/ml for the recovery of intermediate density lipoprotein (IDL) by addition of 3 ml NaCl (71.74 g/l), to 1.063 for low-density lipoprotein (LDL) by addition of 3 ml NaCl (245 g/l) and to 1.210 g/ml for high-density lipoprotein (HDL) by addition of 3 ml 720 g/l of sodium bromide solution. It is convenient to perform the extraction in the presence of 5α-cholestane (25 μl of a 0.025 g/l) added as internal standard to the glass extraction tubes.

Others have used specially devised methods to shorten the time of lipoprotein preparation and thus the exposure to potential peroxidation, including a rapid single discontinuous density gradient ultracentrifugation in a vertical rotor [22–24]

Hodis et al. [19] employed a mild saponification procedure [25], which avoided decomposition or hydrolysis of cholesterol oxides. In brief, the dried lipids were suspended in 20% KOH in methanol and pure diethyl ether (distilled), sealed under argon, and mixed for 3 h in the dark at room temperature (22°C) in a shaker tray (120 oscillations per min). The mixture was neutralized with 20% acetic acid and extracted twice with pure diethyl ether. The pooled sample was then evaporated to dryness under argon and derivatized with diazomethane for 15 min at room temperature (22°C) as described previously [25]. Samples were then evaporated to dryness under argon.

The dried samples were converted to corresponding O-TMS ether derivatives after being sealed under argon and heated at 80°C for 1 h with 0.25 ml of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and

0.25 ml of dimethylformamide (DMF). Aliquots (1 μl) were injected into the gas chromatograph for analysis. All extraction and derivatization steps were performed under subdued light.

Hodis et al. [19] performed their GLC analysis on a Shimadzu GC-14A gas chromatograph fitted with a DB-1 capillary column (30 m×0.25 mm I.D., 0.25 μm film thickness) and a flame ionization detector. Helium was used as the carrier gas at a flow-rate of 1.0 ml/min. The sample was split 20:1. Temperature programming was from 250 to 290°C at 3°C/min with a 32 min hold at the final temperature. The detector and injector temperatures were 300 and 275°C, respectively. Quantitative analysis was performed with an internal standard method (5α-cholestane) utilizing calibration curves, which were linear over the concentration ranges analyzed in this study. GC–MS analysis of samples was performed with a Hewlett-Packard 5985A quadrupole mass spectrometer coupled to a Hewlett-Packard 5840 gas chromatograph through an all-glass jet separator. Temperature of the transfer line, ion source, and jet separator was maintained at 200°C. Ionization current was maintained at 300 μA and the electron energy set at 200 eV. For chemical ionization, the reagent gas utilized was ammonia. Fig. 4A shows a GLC profile of the plasma sterols from a normocholesterolemic rabbit fed standard rabbit chow, while Fig. 4B shows the GLC profile of the atherosclerotic aorta from a hypercholesterolemic rabbit fed a 1% cholesterol diet for 6 weeks [19]. The major cholesterol oxides identified were 7α-hydroxycholesterol, 3,5-diene, 7β-hydroxycholesterol, α-epoxide, and cholestane-triol. After 6 weeks, the cholesterol-fed group showed a significant increase as well as 7α-hydroxycholesterol, 3,5-diene, 7β-hydroxycholesterol, α-epoxide, and cholestane-triol.

The oxosterols identified in the aortic tissue from normocholesterolemic animals were 7α-hydrocholesterol, 3,5-diene, 7β-hydroxycholesterol, α-epoxide, and cholestane-triol. Note presence of β-epoxide, 25-hydroxycholesterol, and 7-ketocholesterol not present in aortic tissue from normocholesterolemic rabbits. Monitoring of the high-cholesterol feed revealed that approximately 0.7% of the cholesterol was oxidized to 3,5-diene, 7α-hydroxycholesterol, 7β-hydroxycholesterol and cholestane-triol. This likely represents the maximum amount of cholesterol

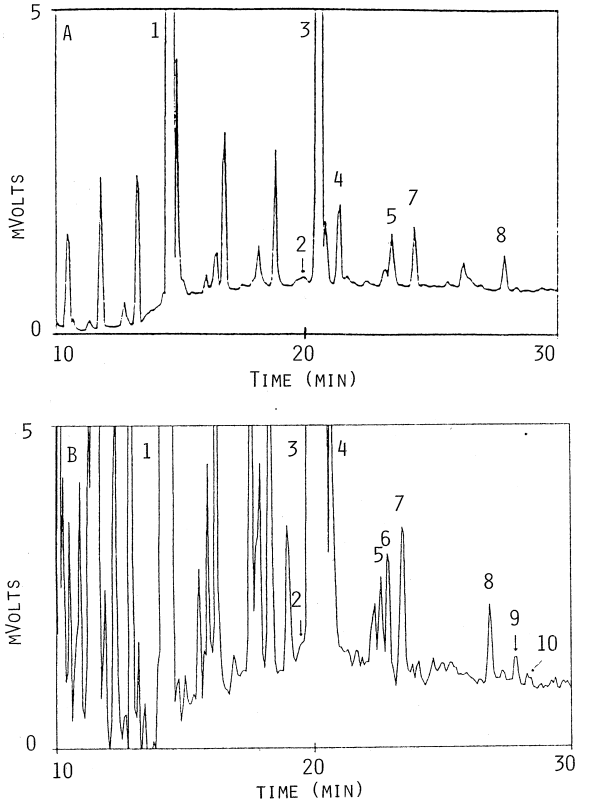

Fig. 4. Gas chromatographic analysis of plasma from a normocholesterolemic rabbit fed standard rabbit chow. Peaks: 1, 5α-cholestane (internal standard); 2, 7α-hydroxycholesterol; 3, cholesterol; 4, 3,5-diene; 5, 7β-hydroxycholesterol; 7, α-epoxide; 8, cholestane triol. GLC conditions: Shimadzu GC-14A gas chromatograph equipped with a DB-1 capillary column (30 m× 0.25 mm I.D., 0.25 μm film thickness, J&W Scientific) and a flame ionization detector. Carrier gas, He at 1.0 ml/min. Temperature programmed from 250°C at 3°C/min with 32 min hold at the final temperature. Detector and split injector temperatures were 300°C and 275°C, respectively. Reproduced from Ref. [19] with permission.

oxidation products ingested by the animals, since these oxidation products formed over a 3-day period of exposure to ambient air in the feeding bins, which infrequently contained enough for 3 full days.

Osada et al. [26] have observed that the absorption rate of individual oxidized cholesterols in the rat differed considerably and was approximately 30% for 7α-hydroxycholesterol, 42% for 7β-hydroxycholesterol, 32% for 5β-epoxycholesterol, 28% for 5α-epoxycholesterol, 15% for cholestanetriol, and 12% for 7-ketocholesterol. Approximately 35 and

48% of cholesterol was recovered in chylomicrons and VLDL, respectively. In contrast, 54 and 40% of the oxidized cholesterol were recovered in chylomicrons and VLDL, respectively, but there was a significant difference in the distribution of individual oxidized cholesterols. The results of this study showed that oxidized cholesterols are absorbed to a lesser extent than is cholesterol (Fig. 5). During the lymphatic absorption the oxidized sterols became esterified to a somewhat lower extent and were distributed differently between the chylomicrons and VLDL. Most of the oxidized cholesterol appeared in the non-esterified sterol fraction of the lymph. Osada et al. [27] have since developed a rapid method of analysis of oxidized cholesterol derivatives by high-performance liquid chromatography (HPLC) combined with diode array UV and evaporative laser light-scattering detection. The resolved oxysterols were identified by reversed-phase LC–electrospray ionization (ESI) MS.

Vine et al. [28] have reported the distribution of oxysterols, including 7-ketocholesterol, 7β-hydroxycholesterol, 5α,6α- and 5β,6β-epoxycholesterols, cholest-4-ene-4-one and 25-hydroxycholesterol, in plasma lipoproteins of rabbits and have assessed whether dietary oxysterols promote oxidative modification of pro-atherogenic lipoproteins in vitro. The oxidized cholesterol-fed animals showed five times the concentration of 5α,6α-epoxycholesterol and double the level of 7-ketocholesterol in triacylglycerol-rich lipoproteins compared to purified cholesterol-fed animals. The presence of 7-ketocholesterol in LDL was exclusive to animals fed the oxidized cholesterol diet. It was concluded that dietary oxysterols may substantially increase the atherogenicity of lipoproteins. In contrast, Lyons et al. [29], in the first report of the distribution of an oxysterol relative to cholesterol, administered simultaneously in a whole animal model, found that [^{14}C]7-ketocholesterol is rapidly metabolized and excreted by the liver. This suggested that diet may not be a major source of oxysterols in atherosclerotic plaque, and that perhaps dietary oxysterols make little or no contribution to atherogenesis. The ^{14}C-labeled metabolites of 7-ketocholesterol were detected by TLC and audioradiography of the lipid extracts. The mobile phase was comprised of hexane–ethyl acetate (1:1, v/v). An excellent separation of 7β-OH (top) and 7α-OH (bottom) cholesterols was obtained. Nevertheless, Rong et al. [30], working with rabbits, and Staprans et al. [31,32], working with LDL receptor and apoE-deficient mice, have obtained evidence that oxidized cholesterol may play an important role in inducing formation of early atherosclerotic lesions. Rong et al. [30] made 20 injections of an oxycholesterol mixture of a composition similar to that found in circulating oxidatively modified LDL. The oxidized cholesterols typically contained 7β-hydroxycholesterol (10%), β-epoxy (25%) and α-epoxy (20%) cholesterols, cholestanetriol (9%), 7-ketocholesterol (35%) and 25 hydroxycholesterol (1%). In Staprans and co-workers [32] experiments, mice were fed regular mouse chow to which 1.0% cholesterol was added (control diet). The oxidized cholesterol diet was identical except that it contained 5% of the added cholesterol was oxidized. The oxidized sterols produced by heating cholesterol as a thin film at 100°C, contained 7–10% 7α-cholesterol; 15 to 20% 7β-hydroxycholesterol; 15–20% β-epoxycholesterol; 10–15% 7α-epoxycholesterol; 40–45%

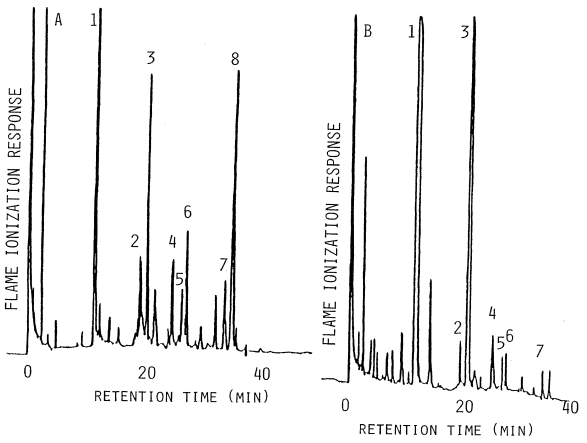

Fig. 5. Gas chromatographic analysis of cholesterol oxidation products administered to rats (A) and oxidized cholesterols recovered in lymph (B). Peaks: 1, 5α-cholestane (internal standard); 2, 7α-hydroxycholesterol; 3, cholesterol; 4, 7β-hydroxycholesterol; 5, 5β-epoxycholesterol; 6, 5α-epoxycholesterol; 7, cholestanetriol; and 8, 7-ketocholesterol. GLC conditions: Shimadzu GC-7AG gas chromatograph equipped with a fused-silica capillary ULBON HR-1 column (50 m×0.25 mm I.D. with 0.25 μm film thickness) and a flame ionization detector. The oven and injector temperatures were 280°C and 300°C, respectively. Carrier gas was helium at 2.2 ml/min. Reproduced from Ref. [26] with permission.

7-ketocholesterol; and a trace of 25β-hydroxycholesterol.

It is possible that some of the results are compromised by inadequate methodology. With regard to saponification, the structural integrity of two types of cholesterol oxides, α-epoxide and 7-ketocholesterol, has been a concern. Contrary to the earlier observation of no structural alteration of β-epoxide [33], an extensive loss of α-epoxide, up to 75%, after conventional hot saponification has been reported, implying that a ring opening was caused by the hot alkaline reaction [34]. Park and Addis [35] have observed that the recovery of α-epoxide was virtually complete when saponification was conducted at ambient temperature for 18 h (cold saponification). The instability of 7-ketocholesterol in hot alkaline medium has long been known, with its dehydration product, cholest-3,5-dien-7-one, being the predominant degradation product [33,36] Thus, Smith et al. [36] used cold saponification to concentrate cholesterol oxides, including 7-ketocholesterol in human liver and plasma samples. Because 7-ketocholesterol is frequently the predominant product of cholesterol oxidation, it is important to accurately determine 7-ketocholesterol to evaluate the extent of cholesterol oxidation in food and biological specimens. Park et al. [37] have reported kinetic data, which demonstrate the extensive destruction of 7-ketocholesterol during conventional hot saponification, but its virtually full recovery from cold saponification (methanolic alkali).

Briefly, aliquots of the stock solution, equivalent to 250 μg of 7-ketocholesterol and 118 μg of 5α-cholestane, were transferred into PTFE-liner screw-cap tube (150×26 mm) to prepare five groups of samples (duplicates). After evaporating ethyl acetate under a nitrogen stream, 10 ml of 1 *M* KOH in methanol was added. After capping the tube, one group was left at room temperature (21°C) for 18 h for cold saponification [35]. The remaining four groups were placed in water baths at 45, 65 and 75°C. Non-saponifiables and internal standard were extracted as previously described [35]. The organic phase was collected, and the aqueous phase was reextracted twice. The solution was backwashed with 0.5 *M* KOH, followed by 5 ml distilled water, and dried over sodium sulfate.

The dried extracts (nonsaponifiables) were redissolved in 50 μl pyridine to which 50 μl of *N,O*-bis(trimethylsilyl)acetamide+trimethylchlorosilane+trimethylsilylimidazole (BSA–TMCS–TMSI, 3:2:3, v/v) (Sylon BTZ) was added to derivatize cholesterol oxides to corresponding TMS ethers for GLC [35]. Capillary GLC was performed with a Varian Vista 6000 gas chromatograph, equipped with a flame ionization detector. The temperature was programmed over the range 210–250°C at 1.8°C/min. The column was a fused-silica capillary column (15 m×0.232 mm I.D.) with 100% methylsilicone stationary phase (DB-1, 0.1 μm film thickness).

GC–MS was performed with a Hewlett-Packard 5890 Series II gas chromatograph interfaced to a 5971A mass-selective detector was used with a DB-5 column (30 m×0.25 mm, 0.1 μm thickness). The column was kept at 270°C, the injector at 280°C, and helium was the carrier gas at 8 p.s.i. Injection was made at 20:1 split ratio. The electron impact ionization was set at 70 eV, and the source temperature was 190°C. Mass spectra scanned between mass/charge (m/z) 100 and 600 were recorded on a computer (Vectra OS/20).

According to Dyer et al. [18] the saponification of lipid extracts for 7-ketocholesterol analysis must be performed at room temperature in the dark. Dyer et al. [18] evaporates the chloroform phase to dryness under nitrogen and dissolves the residue in 2.5 ml of cold KOH (1 *M*) in methanol. Samples are flushed with nitrogen and covered with air-tight film. Saponification is allowed to proceed at room temperature and in the dark for 18–22 h. 6-Ketocholesterol, when used as an internal standard, should also be looked at carefully because of potential tautomerization, leading to the formation of two enol isomers when in extended contact with trimethylsilyl derivatization reagents.

The origin of plasma and aortic tissue cholesterol oxides in both the normocholesterolemic and hypercholesterolemic animals remains a matter of speculation. However, baseline plasma cholesterol oxide levels imply an endogenous origin of these products. Endogenous sources of cholesterol oxides which could contribute to basal levels include the action of tissue cholesterol enzymic hydroxylases, epoxidases, and dehydrogenases along with intravascular sources such as bile acid synthesis, or leukocytes which contain 7α-hydroxylase [38,39].

The low levels of 7α-hydroxycholesterol, 7β-hydroxycholesterol, 3,4-diene, and cholestanetriol found in the cholesterol feed may account for the plasma and aortic tissue elevations of these specific cholesterol oxides upon cholesterol feeding. However, an exogenous source is not likely to account for the presence of α-epoxide and β-epoxide in plasma and aortic tissue or for the presence of 7-ketocholesterol and 25-hydroxycholesterol in aortic tissue since they were not present in the feed.

Using a recently developed method based on isotope dilution mass spectrometry [40], Dzeletovic et al. [41] have determined the kinetics of formation of oxysterols during oxidation of LDL by cupric ions or soybean lipoxygenase. The same products, mainly 7- and 5-oxygenated cholesterol, were formed by the two oxidation methods. During the oxidations, preferentially esterified cholesterol was consumed and consumption of polyunsaturated fatty acids and formation of conjugated dienes preceded the appearance of oxysterols. Dzeletovic et al. [41] used TLC for a reproducible qualitative analysis of cholesterol hydroperoxides. Instead of converting the oxysterol fraction to TMS ethers, it was dissolved in 50 μl of toluene and subjected to TLC (silica $F_{254\ nm}$, 20×20 cm, 0.5 mm film thickness). The mobile phase was toluene–ethyl acetate (3:7, v/v). 7-Oxocholesterol was easily detected by its UV absorption. Plates were visualized with sulfuric acid and heat and the cholesterol hydroperoxide spot was recognized by its R_F and by its characteristic grey–green color.

Capillary GLC has been widely used for the determination of cholesterol and plant sterol contents of the consumed food [42] and the neutral steroid and bile acid contents of the feces [43] were determined by capillary gas–liquid chromatography. In separate tests the RSD was about 2.5% for dietary cholesterol and less than 5% for the total amount of excreted neutral steroids and bile acids. Free cholesterol was assayed with a commercially available test kit (Boehringer Mannheim, Germany; catalog No. 3120328). For the assay of total lathosterol duplicate samples of 200 μl serum were mixed each with 1 ml 0.3 *M* NaOH in 90% ethanol, and incubated for 60 min at 37°C. After addition of l ml distilled water, the non-saponifiable lipids were extracted with 4 ml hexane. Campesterol (6.75 μg, Applied Sciences) was added as internal standard to each of the serum samples as it does not normally occur in plasma. The non-saponifiable lipids were converted into their TMS ethers using SIL-PREP (Alltech Associates).

Caboni et al. [44] have described a fast, sensitive HPLC method for the simultaneous determination of cholesterol hydroperoxides and other major oxysterols, using ultraviolet absorption at 210 nm and light scattering as the detection systems. The hydroperoxides were obtained by cholesterol photooxidation, isolated by TLC and mixed with a standard mixture of 10 oxysterols (epoxy, hydroxy, and keto derivatives). Aliquots were directly injected into a 5 μm particle size, 25×0.46 cm I.D. Spherisorb S5 CN normal-phase column, using *n*-hexane–anhydrous ethanol (97:3, v/v) as mobile phase and a flow-rate of 0.8 ml/min. This method allowed, in a single isocratic analysis, the separation and quantification of the primary and secondary cholesterol oxidation products in 30 min. The light scattering detector was particularly useful for the determination of nonderivatized 5,6-epoxides and cholestane-3β,5α,6β-triol. The sensitivity of both detectors was very similar for most of the oxysterols, except for the 5,6-epoxides and 7-ketocholesterol. Fig. 6 shows the normal-phase HPLC resolution of standard cholesterol oxidation products containing 5α,6α-epoxide, 5β,6β-epoxide, 4β-hydroxide, 20α-hydroxide, 7-keto,25-hydroxide, 19-hydroxide, 7α-hydroxide, 7α-hydroperoxide, 7β-hydroperoxide and triol [44]. The analyses were performed with a HPLC system, which included an ERC, Erma Ins. Degasser (Tokyo, Japan), a 20-μl loop Rheodyne injector Model 7125 (Cotati, CA, USA) and a Knauer pump Model 64 (Berlin, Germany). The HPLC instrument was coupled to either a Sedex (Vitry sur Seine, France) light scattering detector or a Knauer variable-wavelength UV detector. The HPLC instrument was fitted with a Spherisorb S5 CN column (25 cm, 5 μm particle size) with either 4.6 or 3.2 mm I.D. The separations were performed under isocratic conditions using a solution of *n*-hexane–anhydrous ethanol (97:3, v/v) as mobile phase with a flow-rate of 0.8 ml/min for the 4.6 mm I.D. column, and a flow-rate of 0.5 ml/min for the 3.2 mm I.D. column. All cholesterol oxidation product identifications were confirmed by GC–MS with an ion trap detector.

3.2. Lipoprotein distribution

Modifications of previous methods were necessary

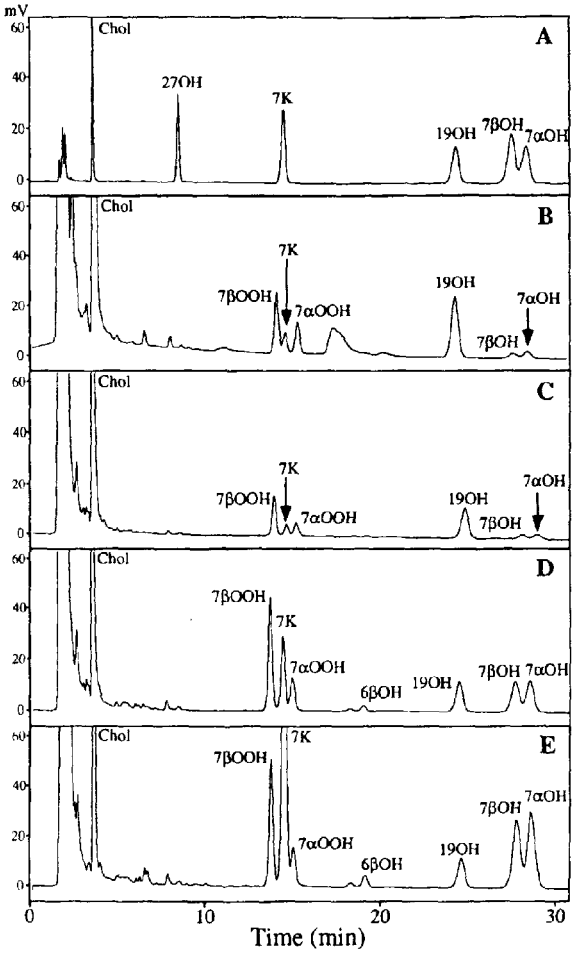

Fig. 6. Normal-phase HPLC analysis of oxysterols. (A) A mixture of sterol standards (2 μg each injected); (B) unilamellar liposomes consisting of egg PtdCho and unesterified cholesterol were oxidized with AAPH (50 m*M*) for 20 h at 37°C; (C) LDL (1.0 mg protein/ml) was oxidized with Cu^{2+} (20 μ*M*) for 4 h; (D) LDL (1.0 mg protein/ml) was oxidized with Cu^{2+} (20 μ*M*) for 8 h; and (E) LDL (1.0 mg protein/ml) was oxidized with Cu^{2+} (20 μ*M*) for 24 h. Saponified extracts were analyzed. Peaks are identified as follows: Chol, cholesterol; 27OH, 27-hydroxycholesterol; 7K, 7-ketocholesterol; 19OH, 19-hydroxycholesterol (internal standard); 7βOH, 7β-hydroxycholesterol; 7αOH, 7α-hydroxycholesterol; 7βOOH, 7β-hydroperoxycholesterol; 7αOOH, 7α-hydroperoxycholesterol; 6βOH, 6β-hydroxycholesterol. Detection was at 205 nm while separating was achieved using normal-phase silica columns and a mobile phase of *n*-hexane–isopropanol–acetonitrile (95.8:3.9:0.3, v/v) at a flow-rate of 1.5 ml/min. Reproduced from Ref. [24] with permission.

for sterol extraction from lipoproteins. The addition of lipoprotein samples to vortex-mixed methanol prior to chloroform–methanol extraction procedure was required to produce consistent recovery of lipid from all lipoprotein classes. Straight extraction with chloroform–methanol (2:1) resulted in less than 30% recovery from LDL and HDL. Mixing with vortex-mixed methanol is required for complete delipidation of lipoproteins [45]. The use of diethyl ether for extraction of non-saponifiable lipid was associated with an artificial increase in concentration of 7-ketocholesterol of the order of 400–500%. The use of diethyl ether should be avoided since it contains significant quantities of ether peroxide which is generated on exposure to light. Artificial oxidation could not be avoided if ether was used despite the use of antioxidants and flushing with nitrogen. The problem did not occur if chloroform was substituted for diethyl ether in all extraction stages.

7-Ketocholesterol is the most abundant of the oxidation products of cholesterol [15] and can be used as an indicator of lipoprotein oxidation [46]. Free radical-mediated lipid peroxidation has been proposed as an important step in the development of atherosclerosis and increased levels of products of free radical mediated reactions have been demonstrated in subjects with vascular disease and diabetes mellitus [47,48]. Cholesterol oxidation products are formed by free radical-mediated oxidation of cholesterol which occurs in association with the production of lipid peroxides [46], and have potentially atherogenic and thrombogenic properties [49]. Published methods for the determination of 7-ketocholesterol are complex [50] or not suitable for the measurement of native lipoproteins [51].

Dzeletovic et al. [40] also investigated the possibility of artificial oxidation during lipoprotein preparation using a ^{13}C-labeled cholesterol standard. [3,4-^{13}C]Cholesterol (1 mg), was added to eight duplicate LDL samples. In four samples this standard was added before, and in 4 samples, after lipid extraction. The standard contained approximately 0.1% of $^{13}C_2$-labeled 7-ketocholesterol. The concentrations of base ions of 7-ketocholesterol (474.7) at the retention time of 7-ketocholesterol standard in the final mixtures were determined by GC–MS. Quantification was performed in the multiple ion (MI) mode, with a scan-time of 2.15 s. Artificial oxidation of [$^{13}C_2$]cholesterol during the lipid extraction proce-

dure would be detected as an increase in the intensity of the base peak ion of [$^{13}C_2$]7-ketocholesterol, 474.7, in relation to the base peak ion of unlabeled 7-ketocholesterol, 472.7. There was no evidence of oxidation of sterols or formation of 7-ketocholesterol during extraction or centrifugation.

Cardenas et al. [52] have proposed an automatic gas chromatographic determination of the HDL cholesterol and total cholesterol in serum. The new method combines on-line precipitation–filtration, enzymatic hydrolysis, extraction and gas chromatography for the determination of total cholesterol and HDL cholesterol in human serum. VLDL, IDL and LDL are precipitated with sodium phosphotungstate and magnesium chloride; then, the serum is continuously filtered and unprecipitated HDL cholesterol is enzymatically hydrolyzed and finally determined as cholesterol by GLC. Total cholesterol is also determined by direct introduction of the serum into the proposed system. The proposed method was verified by analyzing a lipid control serum with certified contents of HDL cholesterol and total cholesterol. Analyses were carried out on Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector and a 10 m×0.53 mm I.D. fused-silica capillary column coated with a 2.65 μm film of 100% cross-linked poly(dimethylsiloxane) (HP-1). Nitrogen was used as a carrier gas at a flow-rate of 37.8 ml/min. The injector port and the detector temperature were kept at 300°C. The oven temperature was raised from 235°C (2 min) to 250°C (5 min) at 3°C/min..

Babiker and Diczfalusy [53] have determined the transport of 24-hydroxycholesterol, 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid in the circulation and the distribution of these oxysterols in the plasma, in the VLDL, LDL, HDL and lipoprotein-free plasma. An accurate method based on isotope dilution-mass spectrometry with use of individual deuterium labeled internal standards was used. 24-hydroxycholesterol and 27-hydroxycholesterol were found to be associated mainly with HDL and LDL, whereas 3β-hydroxy-5-cholestenoic acid was found predominantly in the lipoprotein-free fraction. While both 24-hydrocholesterol and 27-hydroxycholesterol are present mainly in esterified form in plasma, 3β-hydroxy-5-cholestenoic acid was present as free acid only. Babiker and Diczfalusy [53] have determined the distribution of a number of other oxysterols in plasma and in isolated lipoprotein factions. The authors point out that significant amounts of these oxysterols were formed by cholesterol autoxidation during fractionation of plasma according to the procedure of Havel et al. [54]. It was found that the addition of BHT (50 μg/ml) did not prevent autoxidation and practically identical results were obtained with or without added BHT. It was therefore not possible to calculate the distribution of these oxosterols in different plasma fractions. Table 2 gives the concentrations of oxosterols in LDL samples oxidized in vitro [41]. Nine individual oxosterols were determined in the incubation medium from the different time points by GC–MS using individual deuterated internal standards for the different compounds. There was a negligible formation of the side-chain oxygenated sterols 24- and 26-hydroxycholesterol. As seen from Table 2, the dominating oxosterols formed were the 7-oxygenated

Table 2
Oxysterol concentrations in in vitro oxidized LDL samples

LDL treatment	Oxysterol (μg/ml)						
	7α-OH	7β-OH	7-Oxo	5,6α-Epoxy	5,6β-Epoxy	3,5,6-Triol	25-OH
None	0.03	0.04	0.05	0.02	0.07	0.01	0.01
Cu^{2+} (2 h)	5.7	8.1	13.5	1.0	4.9	0.36	0.22
Cu^{2+} (12 h)	12.7	14.1	37.8	4.4	17.8	0.64	0.56
Lipoxygenase (8 h)	7.0	10.9	10.8	1.0	4.4	0.19	0.25
Lipoxygenase (12 h)	17.6	26.1	25.0	3.2	12.9	0.53	0.52

7α-OH, Cholest-5-ene-3β,7α-diol; 7β-OH, cholest-5-ene-3β,7β-diol; 7-oxo, 3β-hydroxycholest-5-en-7-one; 5,6α-epoxy, 5,6α-epoxy-5α-cholest-3β-ol; 5,6β-epoxy, 5,6β-epoxy-5α-cholestan-3β-ol; 3,5,6-triol, cholestane-3β,5α,6β-triol; 25-OH, cholest-5-ene-3β-25-diol.
The oxosterols were determined by isotope dilution-mass spectrometry using deuterium labeled internal standards as described by Dzeletovic et al. [40]. Reproduced from Ref. [41] with permission.

compounds 7α- and 7β-hydroxycholesterol and 7-oxocholesterol. Among these, 7-oxocholesterol dominated and constituted 60–70% of the 7-oxygenated oxosterols. The formation of the initial oxidation products of cholesterol, 7α- and 7β-hydroperoxycholesterol was quantitatively determined by analyzing samples from the different time-points by TLC and comparing the intensity of the cholesterol hydroperoxide spot upon spraying with sulfuric acid. The TLC plates (Silica $F_{254\ nm}$, 20×20 cm, 0.5 mm thick layer, Merck) were developed with toluene–ethyl acetate (3:7, v/v). 7-Oxocholesterol was detected by its UV absorption. Plates were visualized with sulfuric acid and heat. The hydroperoxide spot had a characteristic R_F value and its gray–green color.

Table 3 gives the estimates for 10 different oxosterols in plasma and in VLDL, LDL, HDL and lipoprotein-free plasma after fractionation of the original plasma [53]. The major part of the oxosterols was found in the lDL and HDL fractions except cholestenoic acid which was present to more than 95% in the lipoprotein-free plasma. The VLDL fraction contained very low concentrations of all oxosterols. Table 3 shows that for many of the oxosterols, the sum of the contents in the VLDL, LDL, HDL and LFP fractions were found to be considerably higher than the amount present in the original plasma.

3.3. Significance

Measurement of lipoprotein oxidation may be important in the investigation of atherosclerosis [55-57]. Many methods have been used for the measurements of free radical mediated lipoprotein oxidation, but few are sufficiently sensitive or specific for use in clinical studies. The method of Dzeletovic et al. [40] has the advantage of allowing measurement of 7-ketocholesterol in individual lipoprotein fractions, which may be more informative than the study of whole plasma [56]. The method is sufficiently sensitive to allow measurement of 7-ketocholesterol in native lipoprotein fractions as well as the products of lipoprotein oxidation produced artificially in in vitro systems [46]. Specificity is excellent, in contrast to other methods [48] and confirmation of the identity of 7-ketocholesterol is possible using GC–MS.

Cholesterol oxidation products (oxosterols) have been implicated in several aspects of atherogenesis. They affect key enzymes in cholesterol homeostasis, induce calcification in vascular cells and possess cytotoxic properties [40,41]. Furthermore, plasma accumulation of oxosterols either from enzymic or chemical oxidation has come to be associated with other abnormalities in plasma lipid and lipoprotein metabolism, including inhibition of cholesterol and bile acid biosynthesis, as well as abnormal clearance and accumulation of the LDL by macrophages and

Table 3
Content of oxysterols in isolated lipoprotein fractions (VLDL, LDL, HDL), lipoprotein-free plasma (LFP) and unfractionated plasma (plasma)

Oxysterol	Ng/ml equivalent of plasma						
	VLDL	LDL	HDL	LFP	Sum	Plasma	% SP*
7α-Hydroxycholesterol	2	32	13	5	52	44	120
7β-Hydroxycholesterol	1	15	7	4	27	8	355
Cholesterol-5,6α-epoxide	2	17	8	6	32	9	339
Cholesterol-5,6β-epoxide	4	60	26	16	106	30	53
Cholestane-3β,5α,6β-triol	1	8	3	9	21	21	100
7-Oxocholesterol	1	16	7	8	33	12	266
24-Hydroxycholesterol	3	33	31	12	79	83	96
25-Hydroxycholesterol	1	5	2	4	12	7	166
27-Hydroxycholesterol	3	62	75	18	158	159	100
Cholestenoic acid	0	2	4	118	123	118	105

Sum is the calculated sum of VLDL, LDL, HDL and LFP. Mean of seven samples (from seven individual subjects, sample volume 1.0 ml). *(Sum/Plasma)·100.
The oxysterols were quantified by GC–MS of the TMS ethers obtained by saponification in the presence of internal standard. Reproduced from Ref. [53] with permission.

infiltration of blood vessel walls. The increases in the uncommon plasma sterols while highly significant nevertheless may represent only minor components of plasma sterols, which require highly sophisticated methodology for their detection, identification and quantification.

The ubiquitous presence of cholesterol oxides in human plasma [58] and aortic atheromatous plaques [19] has led to the suspicion that oxosterols may be involved in the initiation and/or progression of atherosclerosis. Studies specifically addressing the issue have shown that oxidative products of cholesterol possess several characteristics which may contribute to their atherogenicity. In addition, cholesterol oxides are toxic to cells. As a result, there is much interest in the determination of cholesterol oxide levels in plasma and plasma lipoproteins as indicators of lipoprotein oxidation and atherosclerosis

Hodis et al. [19] have used GLC and GC–MS to investigate the relationship between plasma and arterial wall oxosterols, and plasma and aortic tissue from seven New Zealand While rabbits fed a high cholesterol (1%) diet for 6 weeks. Cholesterol and cholesterol oxide fractions were isolated and analyzed by GLC. Normocholesterolemic plasma and aortic tissue contained low levels of cholest-5-ene-3β,7α-diol; cholesta-3,5-dien-7-one, 5,6α-epoxy-5α-cholestan-3α-ol, cholest-5-ene-3β,7β-diol, and 5α-cholestane-3β,5,6β-triol while hypercholesterolemic plasma and atherosclerotic aorta contained significantly higher levels of these products. Furthermore, 5,6-epoxy-5α-cholestan-3β-ol not found in normocholesterolemic plasma or aortic tissue, was found in substantial amounts in both hypercholesterolemic plasma and atherosclerotic aortic tissue. Cholest-5-ene-3β,25-diol and 3β-hydroxycholest-5-en-7-one not present in normocholesterolemic aorta was present in the atherosclerotic aorta. The findings demonstrate that there is a strong relationship between plasma and aortic arterial wall levels of cholesterol oxides and suggest that in addition to exogenous sources, formation of cholesterol oxides proceeds via free radical oxidation upon elevated cholesterol levels resulting in the accumulation of these potentially cytotoxic and atherogenic products.

However, in an attempt to test the hypothesis that dietary and plasma oxosterols eventually accumulate in aorta, Lyons and Brown [59] delivered in a physiologically relevant vehicle, chylomicron remnant-like emulsion, to mice and showed that 7-ketocholesterol in chylomicron-remnant like particles is rapidly metabolized and excreted and does not accumulate in aorta. In view of the conversion of 7-ketocholesterol to cholesterol, the authors wonder if the cholesterol accumulating in the aorta could not have originated in 7-ketocholesterol after all.

4. Plant sterols

Small amounts of plant sterols are commonly detected in human and animal plasma and presumably reflect absorption from the diet. Little or no significance is being attached to such findings unless the plant sterol levels exceed several mg%, or are accompanied by the saturated plant sterols. The plant sterols are subject to a differential mucosal absorption. Recently, saturated plant sterols in the form of steryl esters have been including in certain margarines as a means of lowering cholesterol absorption (see below). Plant sterols are esterified to a lesser extent than cholesterol and are not oxidized to normal bile acids by dealkylation and side-chain oxidation. Fig. 7 gives the pathways proposed for the biosynthesis of sterols in vascular plants [60].

4.1. Isolation, identification and quantification

Plasma sterols and steryl esters are usually isolated as part of the total lipid extract obtained by chloroform methanol extraction according to one of the general methods described by Folch et al. [11] and Bligh and Dyer [20]. The plasma and plasma lipoproteins are prepared by conventional ultracentrifugation according to any one of the established procedures [48,61]. The plant sterols in the unsaponifiable matter of dietary oils may be resolved into desmethyl-, methyl- and dimethylsterol fractions by TLC on silica gel H using *n*-hexane–diethyl ether–acetic acid (80:20:0.5, v/v) as the developing solvent [62]. The sterols were visualized by spraying the plate with a solution of dichlorofluorescein and the steroids recovered by elution of the silica gel with chloroform–methanol (2:1, v/v). The fully characterized sterol mixture from rice bran oil may be used as

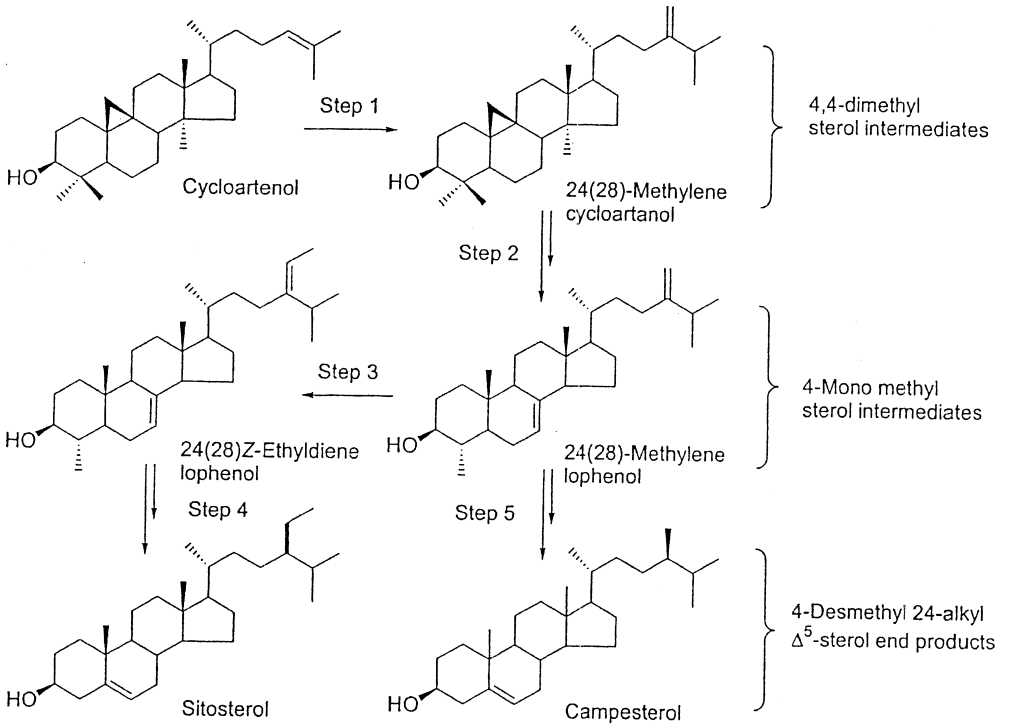

Fig. 7. Pathways of biosynthesis of plant sterols in vascular plants along with chemical structure of selected 4,4-dimethyl and 4-monomethyl intermediates, and 4-desmethyl 24-alkyl Δ^5 end products. Reproduced from Ref. [60] with permission.

a secondary chromatographic reference standard [63].

Fig. 8 shows the GLC resolution of the desmethyl, monomethyl and dimethyl subfractions of rice bran sterols [62], which we have used as a secondary standard for the identification of plant sterols in plasma. The rice bran sterols were resolved into desmethyl-, methyl- and dimethylsterol fractions by TLC on silica gel H using hexane–diethyl ether–acetic acid (80:20:0.5, v/v) as the developing solvent. The plate was visualized by spraying with a solution of dichlorofluorescein and the steroids recovered by elution of the silica gel with chloroform–methanol (2:1, v/v).

Fig. 9 shows a capillary GLC resolution of the free blood plasma sterols from a patient with phytosterolemia before (A) and after (B) oxidative destruction of unsaturated sterols [62]. Even on a 15 m length and over an 8 min period of time a good resolution is obtained with the saturated sterol emerging ahead (separation factor 1.035). Furthermore, the saturated 5β-sterol (coprostanol) emerges ahead of the saturated 5α-sterol, without overlapping with any of the unsaturated sterols of lower molecular mass. This resolution is similar to that reported for other polar liquid phases immobilized packed columns, including SP 1000 [64]. Similar separations are obtained for the saturated and unsaturated C_{28} and C_{29} plant sterols. When the sterols are chromatographed without derivatization, the sterol/stanol separation is even better (separation factor=1.085). A simple but important confirmation of the presence and resolution of the stanols in the sterol mixture may be obtained by rechromatography of the acetates following oxidative destruction of the unsaturated sterols using the method of Serizawa et al. [65].

Since GLC profiles are not as reproducible, when underivatized sterols are chromatographed, Myher and Kuksis [62] recommended acetylation, especially since this approach is compatible with the destruction of the unsaturated sterols prior to rechromatography. In phytosterolemia the stanol peaks emerge

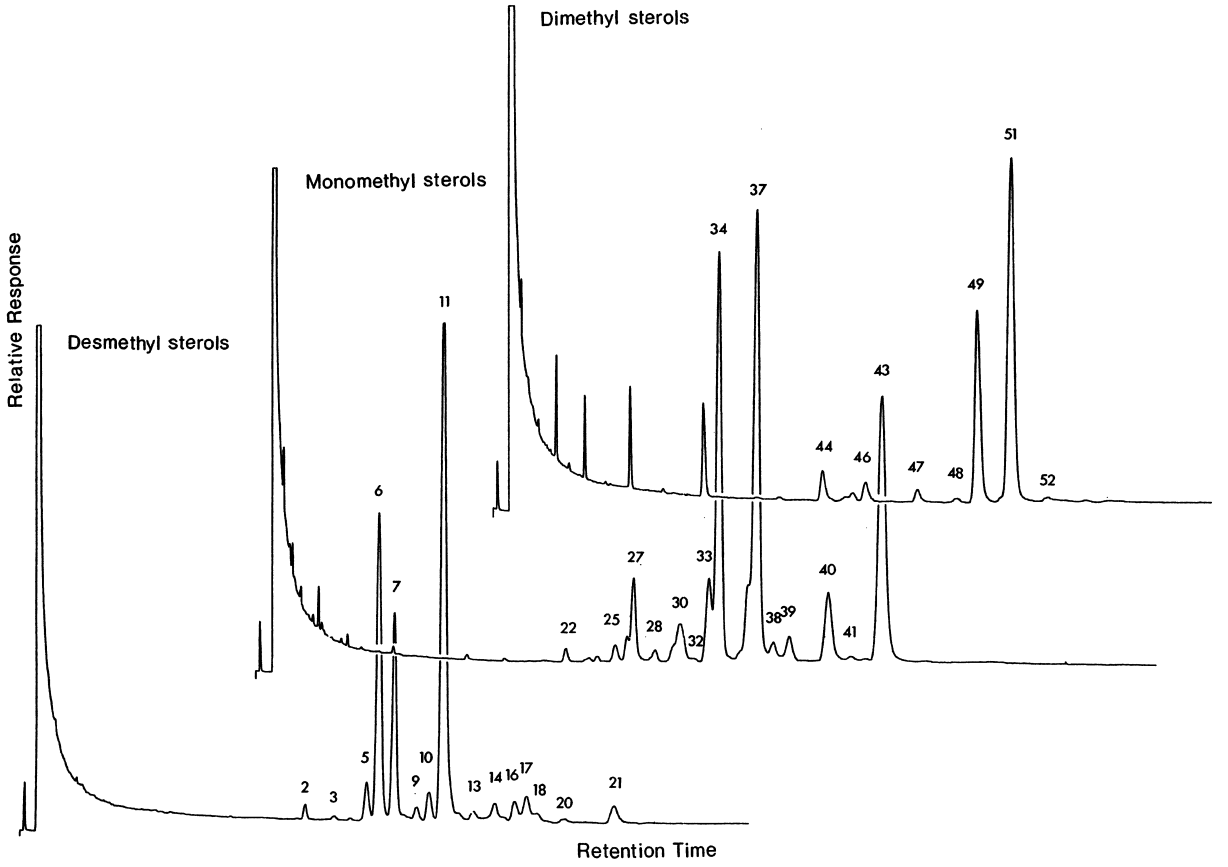

Fig. 8. GLC resolution of desmethyl, monomethyl and dimethyl subfractions of rice bran sterols (as acetates). Peaks are identified as follows: (*desmethylsterols*): 2, cholesterol; 3, brassicasterol; 5, campestanol; 6, campesterol; 7, stigmasterol; 9, 24-methylenecholesterol; 10, stigmastanol; 11, sitosterol; 13, $\Delta^{7,22}$-stigmastanedienol; 14, $\Delta^{7,22}$-methylene cholesterol; 16, Δ^{5}-avenasterol; 17, Δ^{7}-stigmastenol; 21, Δ^{7}-avenasterol; other peaks were not identified; (*monomethylsterols*): 25, campesterol; 27, obtusifoliol; 30, sitosterol; 33, methyl lophenol; 34, cycloeucalenol; 37, 24-methylenelophenol; 39, ethyl lophenol, 43, citrostadienol; other peaks were not identified; (*dimethylsterols*): 46, cycloartanol; 49, cycloartenol; 51, 24-methylenecycloartanol; other peaks were not identified. GLC conditions: Supelcowax 10 flexible quartz capillary column (15 m×0.32 mm I.D.), installed in a Hewlett-Packard Model 5880 gas chromatograph equipped with a flame ionization detector. The carrier gas was hydrogen with 2–5 p.s.i. head pressure. Column temperature was isothermal at 250°C, while the split injector and detector temperatures were 270°C. Reproduced from Ref. [62] with permission.

as clearly resolved components despite a 50–100-fold excess of the Δ^{5}-sterols. Salen et al. [66] has obtained on Chrompack CP-Wax-57CB capillary column a resolution of saturated and unsaturated sterols, which is closely comparable to that just described for the polar capillary column used by Myher and Kuksis [62].

Fig. 10 gives a schematic representation of the GLC retention relative to 5α-cholestane for TMS ethers of unsaturated C_{27} sterols and related sterols on DB-5 (60 m) and CP-Wax columns [67]. GLC retention times for authentic samples of unsaturated C_{27} sterol derivatives were determined using capillary columns coated with D-1 (60 m×0.25 mm I.D.; 100% methyl polysiloxane; 0.1 μm film thickness, J&W Scientific, Folsom, CA, USA), DB-55ms (30 m or 60 m×0.25 mm I.D.; 5% phenyl–95% methylsiloxane; 0.1 μm film thickness; J&W Scientific); Stabilwax (30 or 60 m×0.25 mm I.D.; polyethylene glycol; 0.1 μm film thickness; Restek, Bellefonte, PA, USA); CP-Wax 57CB (25 m×0.32 mm I.D.; high-polarity polyethylene glycol; 0.2 μm

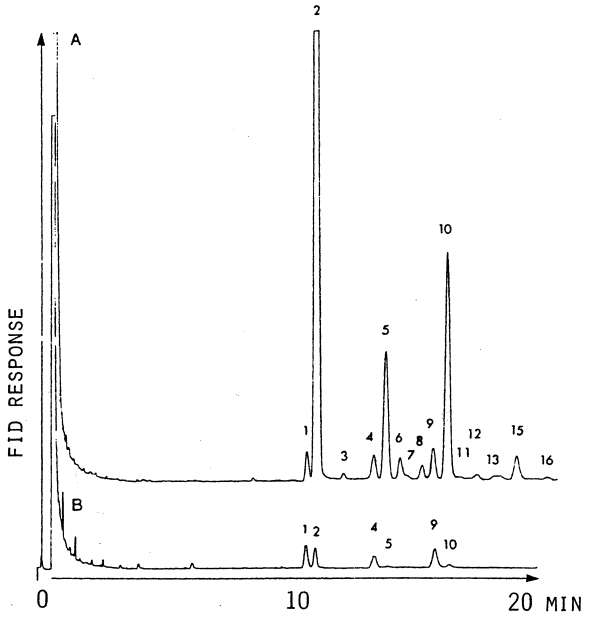

Fig. 9. GLC resolution of blood plasma sterols (as acetates) from a patient with phytosterolemia before (A) and after (B) oxidative destruction of unsaturated sterols. Peaks are identified as follows: 1, 3β-cholestanol; 2, cholesterol; 3, brassicasterol; 4, campestanol; 5, campesterol; 6, stigmasterol; 8, 24-methylenecholesterol; 9, stigmastanol; 10, sitosterol; 15, Δ^5-avenasterol; other peaks were not identified. GLC conditions were as given in Fig. 8. Column temperature, 250°C, isothermal. Reproduced from Ref. [62] with permission.

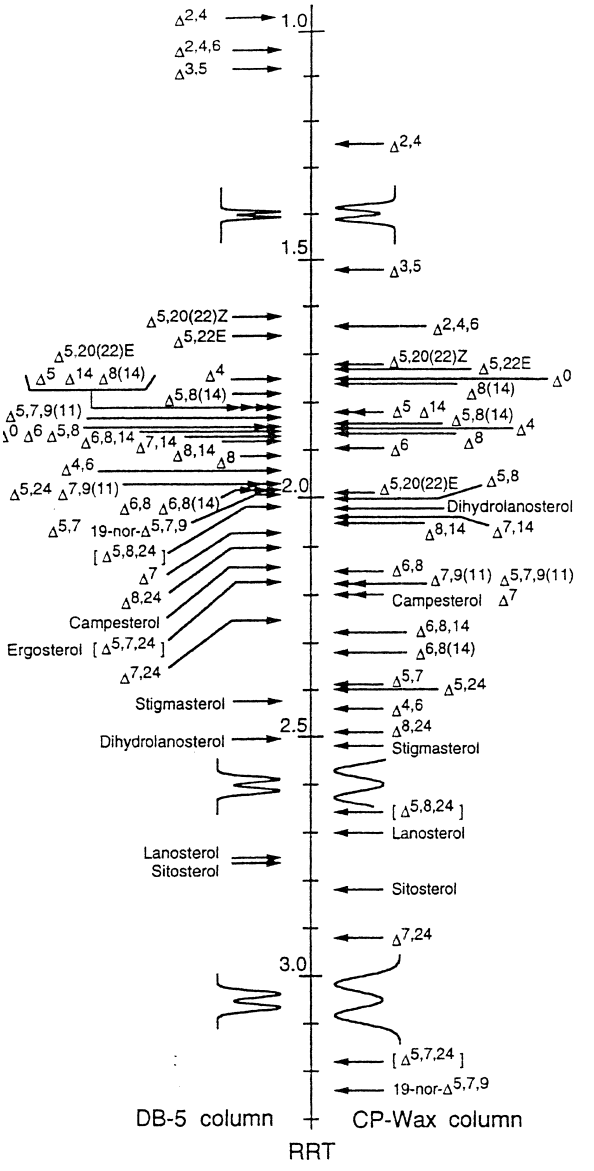

Fig. 10. Retention times relative to 5α-cholestane for TMS ether derivatives of unsaturated C_{27} sterols and related sterols on DB-5 (60 m) and CP-Wax columns. Retention data for the $\Delta^{5,8,24}$ and $\Delta^{5,7,24}$ TMS ethers were predicted according observed changes in retention time upon introduction of a Δ^{24}-bond. Simulated Gaussian peaks centered at RRT 1.4, 2.6 and 3.05 show spacing of two non-overloaded components at resolution R_s 1.0. Reproduced from Ref. [67] with permission.

film thickness; Chromapack, Raritan, NJ, USA). The GLC analyses for DB-1, DB-5, Stabilwax and CP-Wax were done isothermally on a Shimadzu GC-9A instrument. Either nitrogen or helium was used as a carrier gas with a split ratio of 50:1 or 20:1. Injector and flame ionization detector temperatures were held at 250°C and 290°C, respectively. GC–MS analyses were done isothermally at 250°C on a Hewlett-Packard 5890A chromatograph. The temperatures of the injector and GC–MS interface were 270°C. Mass spectra (m/z 50 to 700) were measured on a ZAB-HF reverse geometry double focusing instrument at 0 eV and an electron impact ion source (200°C). The accelerating voltage was 8 kV, and the resolution was 1000 (10% valley).

A simple, specific and reproducible capillary column GLC method for the simultaneous determination of campesterol and sitosterol and 7-ketocholesterol in small volumes of human lipoproteins has been proposed by Dyer et al. [18]. The method involves extraction from lipoprotein samples using chloroform–methanol, saponification of sterol esters

using cold KOH, purification and derivatization to trimethylsilyl ethers using BSTFA and 1% trimethylchlorosilane (TMCS). Oxidation is prevented by drying under nitrogen and the use of powerful antioxidants. Separation is achieved using a DB-1 capillary column and a two-stage temperature ramp from 180 to 250°C and detection using a flame ionization detector.

The identity of sterols can be confirmed by GC–MS. Phytosterols and 7-ketocholeseterol are present at low concentrations in all the major lipoproteins. Using [3,4-^{13}C]cholesterol and GC–MS it could be demonstrated that cholesterol oxidation can be prevented during the processing of lipoproteins using this technique. Internal standard, 25 μl of 0.025 g/l 5α-cholestane in chloroform was added to glass extraction tubes. A modified extraction of Folch et al. [11] was performed. Lipoprotein samples (0.4 ml) were added, dropwise, to 2 ml of methanol while vortexing. The mixture was left to stand for 10 min before addition of 4 ml of chloroform and 0.75 ml water. Samples were then mixed for 60 min. The chloroform phase was separated after centrifugation at 1000 *g* for 20 min.

The total lipid extract may be resolved into free sterols and steryl esters by TLC using a neutral lipid system, which retains the polar phospholipids at the origin. A single development with light petroleum (b.p. 30–60°C)–diethyl ether (150:20, v/v) yielded a single band for total free sterols and another single band for the steryl esters when analyzed on silica gel G (Merck) plates (20×20 cm, 250 μm thick layer) [68]. The free sterols and the steryl ester may then be analyzed separately. Total free sterols and steryl esters were isolated from plasma and the lipoprotein fractions by extraction with chloroform–methanol (2:1, v/v) [69].

It is common practice to subject the total lipid extract to saponification and to isolate the sterols from the unsaponifiable fraction. While this approach may provide a short-cut to a limited objective, as will be shown below, it contains the potential of compromising the analyses of the minor sterols, and, of course, destroying any information about the steryl and oxosteryl esters. The total lipid extract may be advantageously subjected to chromatographic separation of the separation of the free sterols and the steryl esters using HPLC, TLC or high-temperature GLC.

Fig. 11 shows the total plasma GLC profile of a patient with phytosterolemia and of a healthy family member [70]. This particular separation was obtained following a conversion of the plasma phospholipids into diacylglycerols and ceramides and a trimethyl-

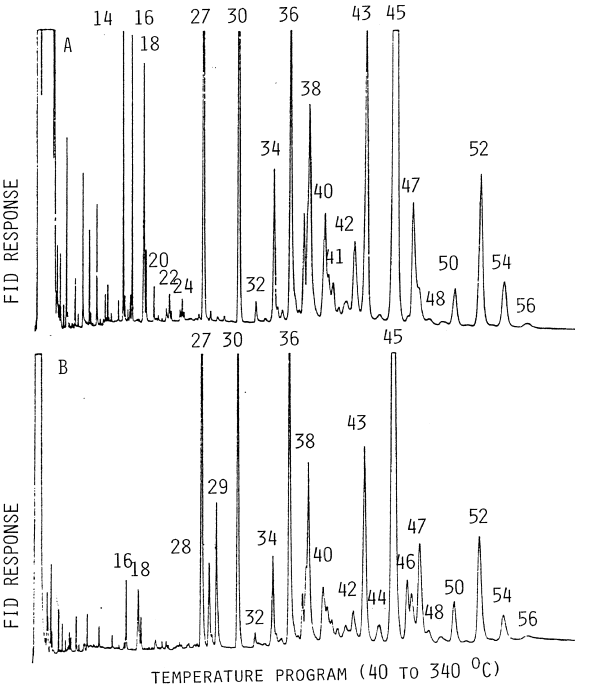

Fig. 11. Total plasma lipid profile of a patient with phytosterolemia (B) and a healthy family member (A) as obtained by high-temperature GLC. Peak identification: 16 and 18, TMS esters of free fatty acids with 16 and 18 carbon atoms; 20, 22 and 24, di-TMS ethers of monoacylglycerols with 16, 18 and 20 acyl carbons, respectively; 27, TMS ether of free cholesterol; 28, TMS ether of free campesterol (arrow); 29, TMS ether of free sitosterol (arrow); 30, tridecanoylglycerol (internal standard); 32–42, TMS ethers of diacylglycerol and ceramide moieties of phospholipids with 30–40 fatty chain carbons; 43–49, steryl esters with 43–49 total carbon atoms (46 is C_{18} fatty acid ester of campesterol and the second peak 47 is the C_{18} fatty acid ester of sitosterol); 50–56, triacylglycerols with 50–56 acyl carbons. GLC conditions: Hewlett-Packard Model 5880A gas chromatograph equipped with an on-column capillary injector and a flame ionization detector. The capillary column (8 m×0.30 mm I.D.) was coated with a permanently bonded non-polar SE-54 liquid phase (Hewlett-Packard). The oven temperature was programmed from 40 to 150°C at 30°C/min, then to 230°C at 20°C/min, to 280°C at 10°C/min and to 340°C at 5°C/min. Hydrogen was the carrier gas at 55.1 kPa head pressure. Plasma samples were digested with phospholipase C and the neutral lipid extracts were trimethylsilylated and diluted with internal standard before analysis. Reproduced from Ref. [70] with permission.

silylation of the sample. The figure shows clearly resolved peaks for free cholesterol, campesterol and sitosterol along with those of the corresponding palmitoyl, oleoyl, linoleoyl and arachidonoyl esters of cholesterol and of the plant sterols, although the latter ones are not as clearly resolved.

We have since obtained identical elution patterns for the free sterols and the steryl esters following a removal of the plasma phospholipids by an adsorption cartridge, and trimethylsilylation of the neutral lipids as shown elsewhere [71]. More recently, Lohninger et al. [72] has shown that total plasma lipid extracts can be injected at low column temperatures and the interference from pyrolysis of phospholipids avoided without removing them prior to GLC.

Plank and Lorbeer [73] have used high-temperature capillary GLC for the determination of the concentration of free sterols as well as their qualitative and quantitative composition and the concentration of the sterol esters in rape-seed oil methyl esters samples by GC–flame ionization detection (FID). Prior to analysis, the free sterols were silylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trichlorosilane; betulinol was used as an internal standard.

The total lipid extract may be resolved into free sterols and steryl esters also by normal-phase HPLC. The free sterol and the steryl ester fractions can then be separately examined for the sterol composition by reversed-phase HPLC, TLC or combinations of these chromatographic techniques with mass spectrometry [69]. Plank and Lorbeer [74] have described an on-line liquid chromatography–gas chromatography system for the analysis of free and esterified sterols in vegetable oil methyl esters used as diesel fuel substitutes. Qualitative and quantitative information about these minor components is provided without saponifcation and off line pre-separation. Prior to analysis the free sterols are silylated with methyl-*N*-trimethylsilyltrifluoroacetamide. Betulinol is used as an internal standard. Using concurrent eluent evaporation with the loop-type interface for eluent transfer, transfer temperatures and transfer efficiency are carefully optimized. The concentration of the free sterols as well as their qualitative and quantitative composition and the concentration of the sterol esters are determined in five different types of vegetable oil methyl esters. The recovery of the LC–GC procedure and reproducibility of the quantitative results are evaluated. Prior to the LC–GC transfer and GC analysis, the large amounts of fatty acid methyl esters were separated from the sterols by LC preseparation. A 100×2 mm I.D. LC column packed with 5 μm silica (Spherisorb, S5W; Phase Separations, Deeside, UK) was used with *n*-hexane–dichloromethane–acetonitrile (79.9:20:0.1, v/v) as the mobile phase at a flow-rate of 350 μl/min. Volumes of 10 μl were injected through a Valco six-port valve; detection was carried out at 220 nm. The column was backflushed with 1 ml of dichloromethane–acetonitrile (95:5, v/v), followed by mobile phase, 3 min after transfer of the LC fraction to the GC system. The backflush valve returned to stand-by 10 min later. Since the elution temperatures of silylated sterols and steryl esters were above 20°C, concurrent eluent evaporation with the loop-type interface was the transfer technique of choice. The GLC separation was carried out on a 12 m×0.32 mm I.D. fused-silica capillary column coated with a 0.1 μm film of 5% phenylpolydimethylsiloxane (DB-5; J&W Scientific), connected in series with a 4 m×0.53 mm I.D. uncoated, deactivated fused-silica precolumn (Carlo Erba), a 3 m×0.32 mm I.D. retaining pre-column coated with DB-5 of 0.1 μm film thickness, and an early vapor exit by means of glass press-fit connection. Carrier gas inlet pressure behind the flow regulator was 250 kPa and the regulated flow-rate was 1.2 ml/min at 40°C (hydrogen). Detector temperature was 370°C. Without going into further details of the exact operation of the system, it may be noted that after 8 min at an initial temperature of 130°C, the GLC oven was heated at 30°C/min to 260°C, then at 3°C/min to 270°C, and finally at 15°C/min to 345°C (held for 7 min). Transfer of 1000 μl took 4.5 min. Total LC–GC run time was 33 min, with 5 min cooling. The free plant sterols were eluted in the order campesterol, sitosterol and brassicasterol clearly dominating. In addition, cholesterol, stigmasterol, and Δ^5-avenasterol could be identified by analysis of samples spiked with reference substances and by comparison to literature data [75]. The later emerging peaks represent the C_{18} fatty acid esters of campesterol and sitosterol. Similar separation of the free plant sterols and plant steryl esters by high temperature GLC had been earlier demonstrated by Kuksis and co-workers [62,70,76] and form part of the well established total lipid

profiling of plasma lipid extracts in health and disease.

Phillips et al. [77] have recently employed a 60 m capillary column with a moderately polar stationary phase (14% cyanopropylphenyl–86% dimethylpolysiloxane) along with a one-step solid-phase extraction to remove highly polar compounds from the saponified total lipid extract. Using epicholesterol as an internal standard, precise quantitative determination of phytosterols, stanols, and cholesterol metabolites in human serum was achieved. Fig. 12 shows representative chromatograms (including epicholesterol internal standard) for reference standards (A and B), Accutrol control serum composite (C), and a sitostanol-containing serum sample from a feeding trial [77]. The chromatograms show unprecedented resolution of the sterols of interest including that of the stanols and the corresponding stenols. Analytes were detected at levels of 120 ng/ml to 6 ng/ml with standard deviations of 0.02 to 0.12 μg/ml. Hidaka et al. [78] have reported a HPLC system that can also distinguish between cholesterol, cholestanol and plant sterols. For this purpose, 0.1 ml of plasma with 10 μg of 5β-cholestane-3α-ol (as an internal standard) was treated with 1 *M* KOH and extracted twice with hexane. The sterols in the extracts were converted into their benzoate derivatives with benzoyl chloride reagent which was freshly prepared for each assay. The benzoate derivatives were re-extracted with 1,2-dichloroethane, and dissolved again in 250 μl of acetonitrile–dichloroethane (2:1, v/v) after evaporation under a stream of nitrogen. The separation of the sterols was performed on a reversed-phase column (SBC-ODS, 150×2.5 mm, Shimadzu, Kyoto, Japan) installed in a Shimadzu LC-6A system and maintained at 50°C and

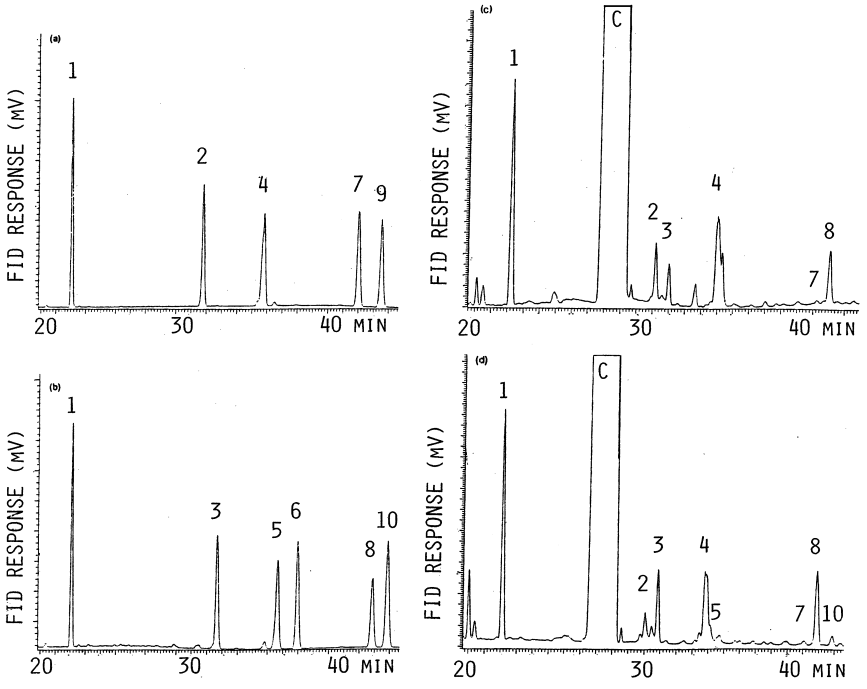

Fig. 12. Representative chromatograms (including epicholesterol internal standard) for reference standards (a and b), Accutrol control serum composite (c) and sitostanol-containing serum sample from a feeding trial (d). Peaks are identified as shown in the chromatograms. Chromatography conditions were as follows: Column, RTx R-171 (14% cyanopropylphenyl–86% dimethylpolysiloxane) (Restek, Bellefonte, PA, USA), 60 m×0.25 mm I.D., 0.25 μm film thickness; carrier gas: hydrogen, 1.18 ml/min (linear velocity, 40 cm/s); split ratio, 9:1 (split vent flow 9.44 ml/min); column head pressure, 21 p.s.i.; injection temperature, 280°C; detector temperature, 280°C; oven temperature, 265°C (45 min), then 10°C/min to 280°C hold 3.5 min. Sample: TMS ethers. Reproduced from Ref. [77] with permission.

monitored at 228 nm. The solvent used for the elution was acetonitrile–water–acetic acid (97:3:0.2, v/v) at a flow-rate of 0.5 ml/min.

Fig. 13 shows the elution pattern of the benzoate derivatives of authentic sterols (A) and plasma sterols from xanthomatosis patient by HPLC [78]. The HPLC system provides an excellent resolution of cholestanol from the corresponding stenols and all other sterols. None of these methods, however, are fully adequate for dealing with the minor sterols, which are sensitive to peroxidation and degradation, and require modification of the conventional methods [67].

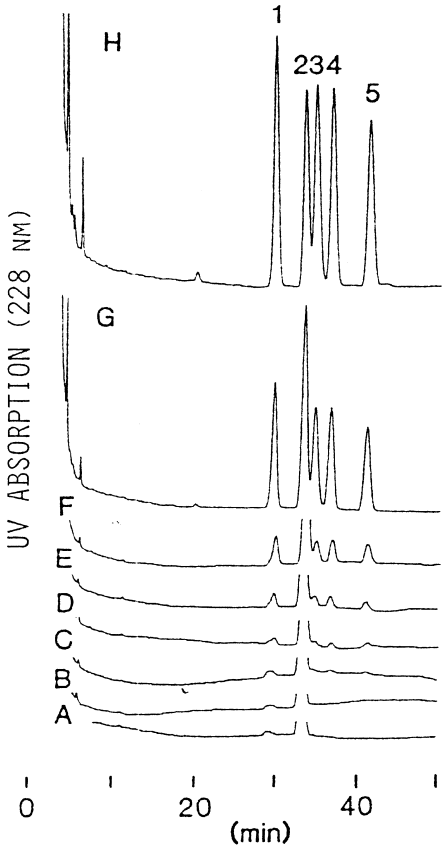

Fig. 13. Elution pattern of benzoate derivatives of authentic cholesterol and plant sterols by HPLC. Peaks: 1, cholesterol; 2, internal standard (5β-cholestan-3α-ol); 3, campesterol; 4, cholestanol; 5, sitosterol. Samples containing 0–10 μg of each sterol and cholestanol with 10 μg of internal standard were derivatized by the same procedure for plasma sterol determination. (A) 0 μg; (B) 0.08 μg; (C) 0.16 μg; (D) 0.31 μg; (E) 0.63 μg; (F) 1.25 μg; (G) 5 μg; (H) 10 μg. HPLC conditions: Shimadzu LC-6A liquid chromatograph equipped with a reversed-phase column (SBC-ODS 150×2.5 mm, Shimadzu, Kyoto, Japan) maintained at 50°C and monitored at 228 nm. The solvent used for elution was acetonitrile–water–acetic acid (97:3:0.2, v/v), at a flow-rate of 0.5 ml/min. Reproduced from Ref. [78] with permission.

4.2. Lipoprotein distribution

It has been suggested that the measurements of phytosterols, campesterol and sitosterol, in human lipoproteins is useful in the study of lipoprotein metabolism. Kuksis et al. [69] have reported LC–MS separation of the mixed steryl esters recovered from LDL/HDL fraction of plasma from a patient with phytosterolemia. Fig. 14 shows the LC–MS elution profile along with the characteristic fragment ions representing the steroid nuclei of cholesterol (*m/z* 369), campesterol (*m/z* 383), stigmasterol (*m/z* 395) and sitosterol (*m/z* 397). From the reversed-phase HPLC column the polyunsaturated steryl esters are eluted first and the less polar saturated esters last. As a result the polyenoic species of both cholesterol and plant sterols are recovered from the HPLC column in somewhat higher proportions than from the polar GLC capillary columns. The chromatogram contains peaks for cholesteryl arachidonate (peak 4), linoleate (peak 9), oleate (peak 17) and palmitate (peak 19), which are seen in the control plasma, as well as readily detectable peaks for the linoleates, oleates and palmitates of campesterol and sitosterol, and small peaks for the minor esters of cholesterol and plant sterols. The peaks were identified from the relative retention times of standards and the identities were confirmed by MS. The parent ions were present in low yields, but in many instances could also be used to establish the identity of the components in a mixed steryl ester fraction. The distribution of the steryl esters is indicated by the ions characteristic of the steroid nuclei, e.g., *m/z* 369 (cholesterol), 383 (campesterol+dihydrobrassicasterol), 395 (stigmasterol+avenasterol) and 397 (sitosterol).

The LC–MS system also allows an examination of each steryl ester peak for the presence of the corresponding 5α-stanyl esters. The presence of cholestanol (*m/z* 371) and stigmastanol (*m/z* 399) could be discerned for each of the major ester peaks and its characteristic retention time (results not shown). The fragment ions of the stanols possess the

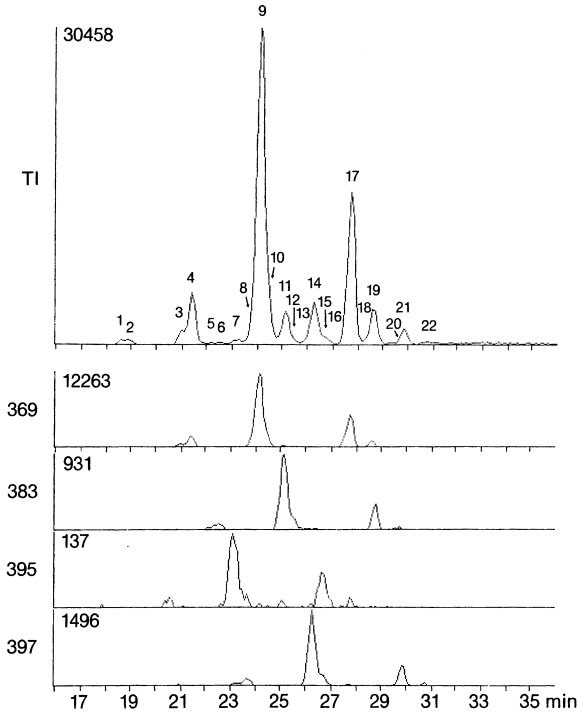

Fig. 14. LC–MS elution profile of steryl esters of the LDL+HDL fraction of plasma from a patient with phytosterolemia. TI, Total ion current; *m*/*z* 369, 383 and 397, fragment ions representing the steroid nuclei of cholesterol, campesterol, stigmasterol and avenasterol, and sitosterol. Peak identification: 1, cholesteryl docosahexaenoate; 2, cholesteryl eicosapentaenoate; 3, cholesteryl linoleate; 4, cholesteryl arachidonate; 7, avenasteryl linoleate; 8, sitosteryl arachidonate; 9, cholesteryl linoleate; 10, cholesteryl palmitoleate; 11, campesteryl linoleate; 14, sitosteryl oleate; 15, avenasteryl oleate; 17, cholesteryl oleate; 19, cholesteryl palmitate; 21, sitosteryl oleate; peaks 5, 12, 13, 16, 18, 20 and 22 were not identified. LC–MS elution conditions: Hewlett-Packard Model 1084B liquid chromatograph equipped with a Supelcosil LC-18 column (Supelco) using a gradient of 30–90% propionitrile in acetonitrile as the eluting solvent. The flow-rate was 1.5 ml/min at 30°C oven temperature. About 1% of the column effluent was admitted to a Hewlett-Packard 5985 B quadrupole mass spectrometer via Hewlett-Packard direct liquid inlet interface. The mass spectra were limited to masses above 200 and were taken every 7 s over the entire elution profile. Reproduced from Ref. [69] with permission.

same mass as the parent ion+2 ion of their unsaturated homologues and must be clearly distinguished from them on the basis of the retention times of the steryl ester peaks. The LC–MS results confirmed the presence of both saturated and unsaturated plant sterols in the various fatty acid ester classes as observed from GLC analyses of the sterols in the $AgNO_3$-TLC fractions of the intact steryl esters (see above). In control plasma only small amounts of the 5α-saturated stanols were found (0.4% of total). In contrast, substantial amounts of the 5α-saturated stanols were present in the phytosterolemia plasma. The LC–MS approach using a single quadrupole did not allow differentiation between the fatty acid esters of campesterol and its isomer 22,23-dihydrobrassicasterol, which were eluted with similar retention times and possessed very similar chemical ionization mass spectra.

Table 4 gives the concentrations of campesterol, sitosterol and 7-ketocholesterol in VLDL, IDL, LDL and HDL of 10 normal men. Concentrations of sterols are expressed as μmol/l of whole serum and also as a ratio to cholesterol concentration of each lipoprotein fraction [18]. Separation of cholesterol, campesterol, sitosterol and 7-ketocholesterol was performed using DB-1 capillary column. Nitrogen (oxygen free) was used as the carrier and auxilliary gas at flow-rates of 1–2 ml/min. The injection split ratio was 3:1. The injector temperature was 270°C and the detector temperature was 300°C. The starting oven temperature was 180°C, which was increased by 8°C/min to 220°C and then with 2°C/min to a final temperature of 260°C. Peak height was used for quantification. Concentrations of cholesterol and

Table 4
Concentrations of phytoserols and 7-ketocholesterol in lipoprotein fractions of 10 normal men

	Campesterol	Sitosterol	7-Ketocholesterol
Concentration of sterol (mmol/l)			
VLDL	0.66 (0.25)	0.37 (0.10)	0.66 (0.49)
IDL	0.79 (0.28)	0.49 (0.16)	0.58 (0.38)
LDL	7.23 (3.20)	3.82 (2.05)	1.97 (1.28)
HDL	2.92 (1.18)	1.78 (0.64)	1.17 (0.55)
Concentration of sterol/concentration of cholesterol* (mmol/mol)			
VLDL	2.02 (1.03)	1.10 (0.51)	1.82 (1.90)
IDL	2.10 (0.93)	1.11 (1.04)	1.19 (0.81)
LDL	2.38 (0.77)	1.23 (0.38)	0.63 (0.34)
HDl	2.00 (0.75)	1.14 (0.42)	0.94 (0.78)

Means (S.D.). *Determined enzymatically.
The concentrations of the plant sterols were determined by capillary GLC of TMS ethers of plant sterols and 7-ketocholestrol using 5α-cholestane as internal standard. Reproduced from Ref. [18] with permission.

other sterols were calculated from standard curves of the appropriate sterol using 5α-cholestane as internal standard.

The limit of quantification was determined by repeated analysis of sterols at low concentrations. The standard deviation of 10 measurements of a number of sterols was calculated. The concentration at which the mean exceeded zero by more than three standard deviations (SDs) was taken as the limit of sensitivity.

Cholesterol, campesterol, sitosterol and 7-ketocholesterol were provisionally identified by their retention times compared with commercially available standards. Their identities were confirmed using GC–MS. Helium was used as carrier gas (2 ml/min flow-rate). The ion sources was held at 80°C and operated in the electron impact (EI) mode. Samples were applied via an on-column injector, with an oven temperature 210–290°C, ramping at 2°C/min. Sterols were identified by their fragmentation pattern while scanning the mass range m/z 200–680.

4.3. Significance (sitosterolemia and xanthomatosis)

There is good evidence that increased levels of plasma sterols other than cholesterol can serve as markers for abnormalities in lipid metabolism associated with clinical disease [66]. Thus, premature atherosclerosis and extensive xantomatosis have been described in two rare lipid storage diseases, cerebrotendinous xanthomatosis (CTX) and sitosterolemia with xanthomatosis. In cerebrotendinous xanthomatosis, severe neurologic dysfunction, cataracts, pulmonary abnormalities and endocrine hypofunction are found, while in sitosterolemia with xanthomatosis, arthritis and episodic erythrocyte hemolysis are noted occasionally. Specifically, in cerebrotendinous xanthomatosis, cholestanol, the 5α-dihydro derivative of cholesterol is present in all tissues with particularly high amounts in brain, nerve, xanthomas and bile. In distinction, large quantities of plant sterols (campesterol and sitosterol) along with 5α-saturated stanols, cholestanol, 5α-campestanol and 5α-sitostanol accumulate in plasma of subjects with sitosterolemia with xanthomatosis. Although the mechanism for the enhanced sterol accumulation is not known, abnormal lipoprotein sterol transport has been suggested to play a role during both sterol absorption and tissue distribution.

Furthermore, plasma accumulation of oxosterols either from enzymic or chemical oxidation has come to be associated with other abnormalities in plasma lipid and lipoprotein metabolism, including inhibition of cholesterol and bile acid biosynthesis, as well as abnormal clearance and accumulation of the low density lipoprotein by macrophages and infiltration of blood vessel walls.

Inhibition of cholesterol biosynthesis is associated with the plasma appearance of the precursor sterols, while inhibition of cholesterol conversion into bile acids is associated with the plasma appearance of the intermediate oxidation products of cholesterol.

The increases in the uncommon plasma sterols while highly significant nevertheless may represent only minor components of plasma sterols, which require highly sophisticated methodology for their detection, identification and quantification. Lipoprotein sterol transport has been suggested to play a role during both sterol absorption and tissue distribution.

Fig. 15 shows the GLC elution profile of steryl esters of VLDL (upper tracing) and LDL+HDL (lower tracing) fractions from a patient with phytosterolemia [69]. There is a complete separation of the corresponding saturated and monounsaturated as well as the di- and polyunsaturated fatty acid esters. From the polar liquid phase the polyunsaturated fatty acid esters are eluted last and suffer some losses. As a result the contribution of the arachidonoyl species is underestimated. In addition to the peaks for the cholesteryl esters, those for corresponding esters of campesterol plus dihydrobrassicasterol, and sitosterol are also readily detected. Table 5 gives the quantitative distribution of the cholesteryl and plant steryl esters among the lipoprotein classes [69]. There is evidence that the plant steryl esters are concentrated mainly in the LDL+HDL fractions and that they may experience some difficulty in transfer to the VLDL possibly via the cholesteryl ester transfer protein.

In separate experiments [79,80], the Δ^5-sterols, cholesterol, campesterol, and sitosterol were separated from their 5α-dihydro derivatives cholestanol, 5α-campestanol, 5α-sitostanol by argentation TLC and then quantified as their TMS ether derivatives by GLC on a 6 ft×4 mm glass columns packed with 3%

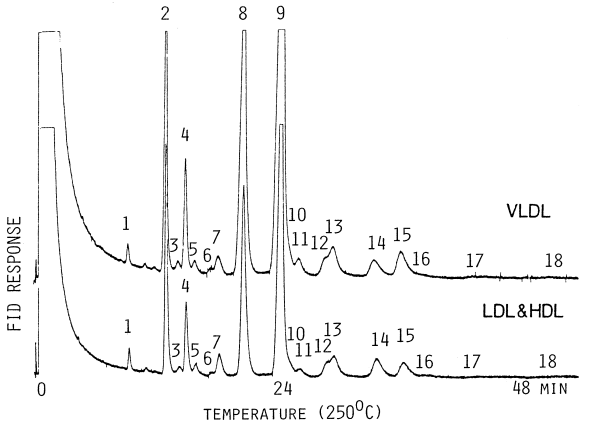

Fig. 15. GLC elution profile of steryl esters of VLDL (A) and LDL+HDL (B) fractions of plasma from a patient with phytosterolemia. Peak identification: 1, cholesteryl myristate; 2, cholesteryl palmitate; 4, cholesteryl palmitoleate; 5, campesteryl palmitate; 7, sitosteryl palmitate+cholesteryl stearate; 8, cholesteryl oleate; 9, cholesteryl linoleate; 10, campesteryl oleate; 12, sitosteryl oleate; 13, campesteryl linoleate; 14, sitosteryl oleate; 15, cholesteryl arachidonate. GLC conditions: Hewlett-Packard gas chromatograph equipped with an SP-2330 glass capillary column (10 m×0.25 mm I.D.) and a hydrogen flame ionization detector. The split injector and detector temperatures were 270°C. Column temperature, 250°C, isothermal; Carrier gas, hydrogen at 8 p.s.i. inlet pressure. Sample intact steryl esters. Reproduced from Ref. [69] with permission.

QF-1 (Applied Science Labs.) (1 ft=30.48 cm). The retention times relative to 5α-cholestane are: cholesterol 1.73, cholestanol 1.85, campesterol 2.52, 5α-campestanol 2.66, sitosterol 3.03, 5α-sitostanol 3.17. It is necessary to separate the unsaturated sterols from their 5α-saturated derivatives by argentation TLC because only small differences exist between the unsaturated and 5α-saturated sterol peak retention times on OF-1. However, quantitative results by the two independent methods agreed within 10%.

Fig. 16 shows a GLC separation of underivatized sterols present in plasma of control, cerebrotendinous xanthomatosis and sitosterolemic patients that illustrates the separation of the Δ^5-unsaturated sterols from their 5α-saturated analogs on the SP 1000 column [66]. The CTX patient's plasma contained significantly more cholestanol than the control subject's and plasma. The plasma of the sitosterolemia patient contained cholestanol, cholesterol, campestanol, campesterol, sitostanol and sitosterol.

Table 5
Quantitative composition of steryl esters of VLDL and LDL+HDL fractions of plasma from a patient with phytosterolemia (mol%)

Peak No.	Steryl ester	Total	Lipoprotein fractions	
			VLDL	LD+HDL
1	Chol. 14:0	0.8	0.6	0.9
2	Chol. 16:0	12.9	11.8	13.6
3	Chol. 16:1(*n*-9)			
4	Chol. 16:1(*n*-7)	4.6	5.0	4.9
5	Camp. 16:0	0.7	0.6	1.1
6	Unknown		0.3	0.4
7	Sito. 16:0+Chol. 18:0	1.9	1.0	2.2
8	Chol. 18:1	23.3	25.3	19.3
9	Chol. 18:2	41.6	44.0	36.5
10	Camp. 18:1			
11	Unknown			
12	Sito. 18:1	1.3	1.3	3.1
13	Camp. 18:2	2.0	1.6	3.8
14	Sito. 18:2	4.2	1.8	5.7
15	Chol. 20:4	3.2	2.9	4.9
16	Chol. 20:3?		0.3	1.0
17	Camp. 20:4	tr	0.3	0.5
18	Sito. 20:4	tr	N.D.	0.4
Other			2.7	1.0

tr, Trace; N.D., not determined. Steryl esters were resolved intact on a polar capillary GLC column as described for plasma total lipid profiles. Reproduced from Ref. [69] with permission.

5. Differential sterol absorption

In contrast to cholesterol, plant sterols are poorly absorbed. From a structural standpoint, non-absorbability is most clearly related to substitutions in position C_{24} [81–83] on the sterol side chain with methyl or ethyl groups or their unsaturated counterparts as exemplified by campesterol (24-methyl), sitosterol (24-ethyl), stigmasterol (Δ^{22}, 24-ethyl), and fucosterol (24-ethylidine). Each of these is poorly absorbed and inhibits cholesterol absorption.

5.1. Mechanism of cholesterol absorption

In the absence of an established mechanism of cholesterol absorption, it is difficult to speculate about the mechanism of plant sterol absorption and a especially about the plant sterol inhibition of cholesterol absorption. Nevertheless, the speculation continues unabated. Recently, the molecular biologists

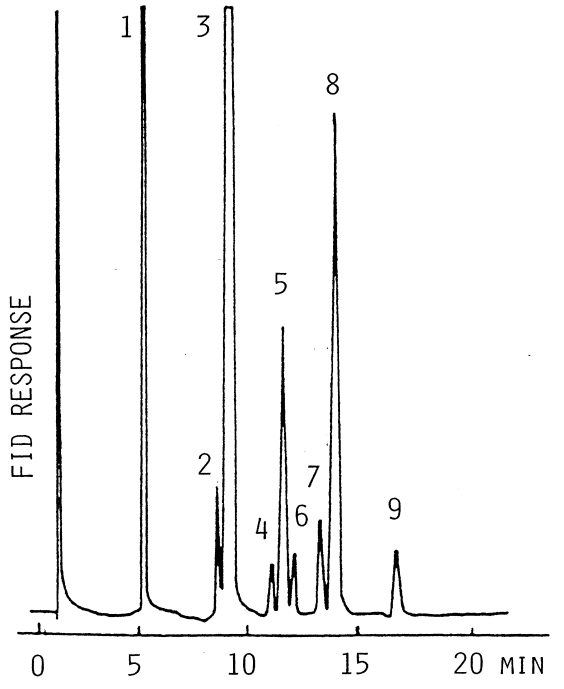

Fig. 16. Illustration of the GLC separation of underivatized saturated and unsaturated sterols from a CTX patient's plasma on an SP 1000 column. Peak identification: 1, 5α-cholestane (internal standard); 2, cholestanol; 3, cholesterol; 4, campestanol; 5, campesterol; 6, stigmasterol; 7, sitostanol; 8, sitosterol; 9, avenasterol. Reproduced from Ref. [66] with permission.

have joined in with first evidence for a role of sterol transport proteins [84].

Early work suggested several potential sites for discrimination, including the level of mucosal sterol esterification [85] and lumenal solubilization [86,87]. Ikeda et al. [86] have presented data to suggest that the mechanism that accounts for inhibition of cholesterol absorption is distinct from that which discriminates between cholesterol and plant sterols for absorption. Two major sites appeared to be involved, namely, differential uptake at the brush border and differential affinity for bile salt in micelles. The possibility that absorbable and unabsorbable sterols possess different affinities for the micelle as suggested by Armstrong and Carey [87], could result in a differential delivery of the sterols to the cell surface.

Several studies support discrimination between cholesterol and sitosterol at the brush border membrane. Child and Kuksis [88], using the 7-dehydro analogs of the two sterols in micellar solution, reported a 4–5-fold uptake differential by rat brush border membranes in vitro, and Ikeda and Sugano [89] reported the intestinal uptake of sitosterol intubated into the stomach of rats was about one fifth that of cholesterol.

Fig. 17 shows the capillary GLC elution patterns of the plant sterols of menhaden oil as the total unsaponifiable matter (bottom), the sterols of the intestinal brush border membrane (middle) and lymph sterols (top) during menhaden oil absorption in the rat [90]. In comparison to the original unsaponifiable matter, the brush border sterols are enriched in campesterol and Δ^7-cholestenol (lathosterol) and sitosterol, specifically discriminating against 24-methylenecholesterol. In contrast to the brush border sterols, the lymph sterols have become enriched desmosterol, while also transferring efficiently the 24-methylenecholesterol that had entered the brush border. However, the transfer of the Δ^7-cholestenol to lymph was strongly impaired, although its transfer from the lumen to the brush border had been favored. This study shows that the sterols of the menhaden oil were subject to three levels of selection: first, at the lumenal solubilization; second at the brush border uptake; and third, at the lymphatic transfer. Previously, Kuksis and Huang [81] had shown that campesterol (24α-methyl) is absorbed into the dog lymph at a rate between that of cholesterol and sitosterol (24β-methyl), while Vahouny et al. [82] later showed that plant sterols with 24-methyl and methylidine substitutions were absorbed more efficiently, but less than cholesterol. Vahouny et al. [82] had also shown a significant lymphatic absorption of brassicasterol (Δ^{22}, 24β-methyl), which is a significant component of bivalve shellfish sterols, at about 9% when assessed after administration of mixed sterols. Because of insufficient concentration in the unsaponifiable matter of menhaden oil, the lymphatic absorption of brassicasterol was not established in the more recent work of Kuksis and co-workers.

Based on recent work with ABC transport proteins by Berge et al. [91] and on other recent studies, Allayee et al. [84] have provided a rationalization for the differential absorption of cholesterol and plant sterols by the intestine. Dietary sterols passively

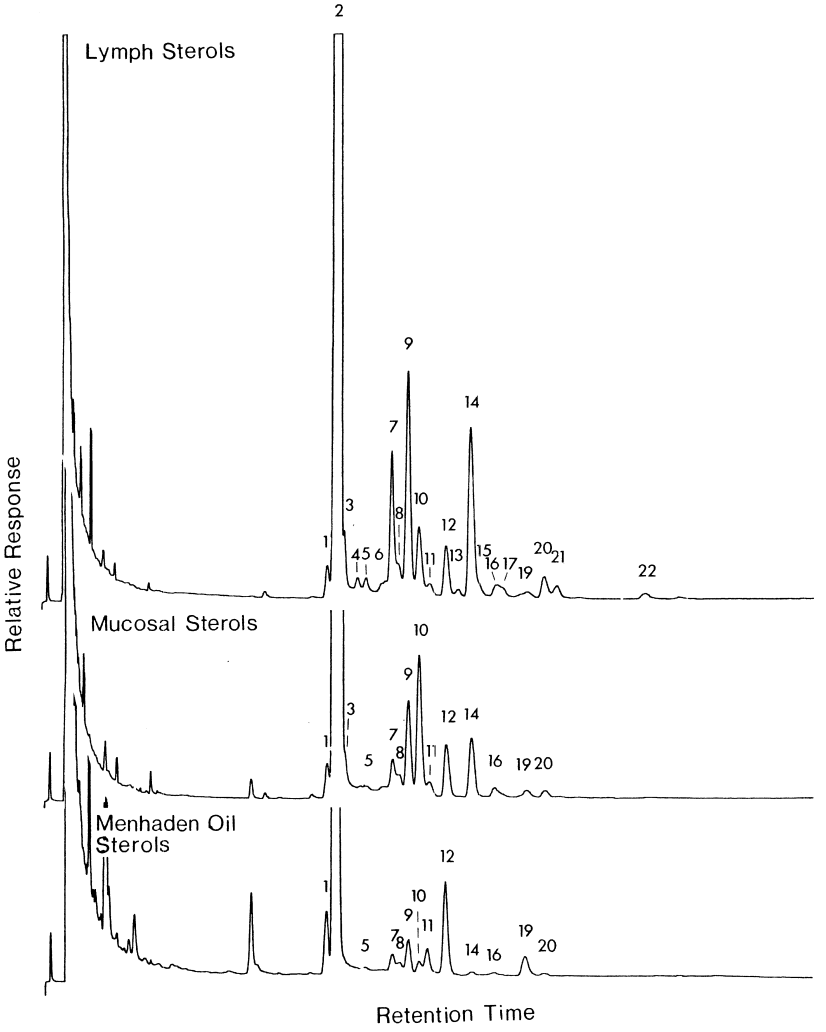

Fig. 17. GLC elution profile of the sterols in the unsaponifiable matter of menhaden oil (bottom), intestinal brush border (middle) and intestinal lymph (top) sterols of rats receiving menhaden oil in the diet. Peak identification is as follows: 1, cholestanol; 2, cholesterol; 4, brassicastanol; 5, brassicasterol; 7, Δ^7-cholestenol (lathosterol); 8, campestanol; 9, campesterol; 10, desmosterol; 12, 24-methylenecholesterol; 13, sitostanol; 14, sitosterol; 15, Δ^7-ergosterol; 16, $\Delta^{5,7}$-ergostadieneol; 18, $\Delta^{7,24}$-methylenecholesterol; 19, Δ^5-avenasterol; 21, Δ^7-stigmasterol; 22, Δ^7-avenasterol; 23, unknown. GLC conditions are as given in Fig. 9 [90].

enter intestinal cells and a proportion of them are actively pumped back into gut lumen by the ABC transporter proteins. Berge et al. [91] used a combination of mapping information and functional data to identify the genes that are defective in sitosterolemia.

Chemical saturation of the Δ^5 double bond leads to the introduction of a 5α-position of the hydrogen atom (campestanol and sitostanol), whereas enzymatic transformation by bacteria in the intestine leads to the 5β-position (methyl and ethyl coprostanols). Several studies have indicated that sitostanol is not absorbed at all [92–94], and feeding of this saturated plant sterol seems to have more pronounced effect on reduction of serum cholesterol than does sitosterol. There is evidence that suggest that the mechanism that accounts for inhibition of cholesterol absorption is distinct from that which discriminates between cholesterol and plant sterols

for absorption. Specifically, sitosterol [95] and fucosterol [86] displaced cholesterol from micellar solution accounting for the inhibition of its absorption, but micellar solubilization does not insure absorption, since sitosterol is not absorbed even when fully micellar-solubilized [86]. Several potential sites for discrimination have been suggested. One is at the level of mucosal sterol esterification and is based on the repeated observations that, in contrast to cholesterol, sitosterol appears largely unesterified in the lymph of experimental animals. This observation is supported by in vitro studies that demonstrate that sitosterol is esterified less well [96,97] than cholesterol by the intestinal esterification enzymes, cholesterol esterase [86] and ACAT [98].

Itoh et al. [75] have investigated the sterol fractions from a number of major vegetable oils by GLC. The unsaponifiables from 19 vegetable oils were divided into sterol and three other fractions by TLC. A sample of 30 mg was applied uniformly along a line 1.5 cm from one edge of the plate and developed with hexane–diethyl ether (8:2, v/v) for 1 h. The plate was sprayed with 0.01% Rhodamine 6G solution in ethanol and observed under UV light (3600 Å). Four separate zones containing less polar compounds, triterpene alcohols, 4-methylsterols, and sterols, respectively, were cut off and extracted with ether. The ether extract from the sterol-containing zones was desiccated for subsequent GLC analysis. The sterol zone was analyzed with a Shimadzu GC-5A GLC system with FID. The chromatograph was fitted with a glass column, 2 m×3 mm I.D., packed with Gas Chrom Z, 80–100 mesh, 250°C with nitrogen at 50 ml/min as carrier gas. Under these conditions the retention time of β-sitosterol was 30 min. GC–MS was done with helium as the carrier gas, a molecular separator, ionizing voltage of 70 eV, trap current 60 μA. Cholesterol, brassicasterol, campesterol, stigmasterol and sitosterol were identified by comparing their relative retention times (RRTs) with those of the reference standards. Other sterols were identified as fucosterol. The presence of an ethylidine group at C-24 is indicated by the peak at m/z 314 which arose from the loss of part of side chain ($-C_7H_{14}$) by a McLafferty rearrangement, and is typical for sterols containing $\Delta^{24(28)}$-bond [99,100]. Knights [99] separated fucosterol and its C_{29} isomer (Δ^5-avenasterol) by GLC using Hi-EFF-8B as polar liquid phase. Other minor sterols were identified as Δ^7-avenasterol, Δ^7-stigmastenol and Δ^5- and Δ^7- were detected in most of the oils. Cholesteryl esters of plant oils were done in our own laboratory by GC–MS. Campesterol, stigmasterol and sitosterol were present in all oils, and a minor amount of cholesterol in the majority of oils. Brassicasterol was found mainly in rapeseed oil.

Alkaline hydrolysis and extraction of cholesterol and plant sterols was used to quantify cholesterol, sitosterol and sitostanol by GLC (as the TMS ethers in presence of 5α-cholestane) for the study of the mechanism of action of plant sterols on inhibition of cholesterol absorption. Complete separation of all the sterols was achieved using the method of Mattson et al. [101]. Overall cholesterol absorption declined during sitosterol infusion by almost 50%, whereas sitostanol infusion caused a reduction of cholesterol absorption by almost 85%. These findings of a more effective inhibition of cholesterol absorption by sitostanol might confirm the observation recorded by others that an increase in hydrophobicity of a plant sterol results in a higher affinity but lower capacity to mixed micelles. This may cause an effective displacement of cholesterol from micellar binding and therefore diminished cholesterol absorption. The sterol uptake was studied in two normal volunteers by intestinal perfusion [102].

The possible involvement of pancreatic cholesterol esterase in the directed sterol uptake and esterification in enterocytes has been investigated by Lopez-Candales et al. [103]. In the presence of cholesteryl esterase, the level of cholesteryl ester increased 39-fold indicating that the enzyme-mediated uptake accounts for 120-fold greater ester synthesis than that from basal absorption. To determine the amount of cholesteryl ester in the cellular debris, 20 μl of 1 m*M* cholesteryl oleate containing [^{14}C]cholesteryl oleate (1000 cpm) were added as carrier and efficiency standard, respectively. The lipids were extracted by adding 1.8 ml of chloroform–methanol (2:1) followed by 0.5 ml of water and 0.5 ml of chloroform. The chloroform layer was removed and evaporated under a stream of nitrogen, and the residue was redissolved in 150 μl of light petroleum–methanol–chloroform (1:1:1, v/v). The lipids were chromatographed on silica gel OF plates using light petroleum–diethyl ether–acetic acid (75:5:1).

After visualization with iodine vapor, the cholesteryl ester spot was scraped into scintillation fluid, and the amount of synthesized [^{3}H]cholesteryl esters was calculated after correction for spillover from the [^{14}C]cholesteryl oleate efficiency standard. The efficiency of extraction was always between 60 and 75%.

5.2. Inhibition of cholesterol absorption

Peterson [104] reported for the first time that the increase of plasma cholesterol levels in chickens caused by cholesterol feeding can be prevented by including 1% soybean sterols in the diet. Since then, numerous studies have confirmed a hypocholesterolemic action of plant sterols, especially sitosterol by Beveridge and co-workers [105,106], Lees et al. [107], Lederle [108], Vanhanen and Miettinen [109] and Gylling et al. [110].

A great renewal has occurred in the use of plant sterols for the inhibition of cholesterol absorption. The novel development was to convert plant sterols to the corresponding stanols and esterify them to fat soluble form [111]. Gylling et al. [112] have developed a sitostanol ester margarine that includes sitostanol in soluble ester form so that the margarine can replace a small amount of normal dietary fat, causing 10–15% reduction in serum total and LDL cholesterol level of mildly hypercholesterolemic adult subjects. Campesterol and sitosterol, plant sterols which may be indicators of dietary cholesterol absorption [113] can be measured within the same analytical run. Measurement of the concentrations of phytosterols may be useful for the study of cholesterol metabolism particularly in large scale epidemiological studies [113]. Concentrations of campesterol and phytosterols are similar to published levels using similar methods, both in whole serum [114,115] and lipoproteins [116].

Miettinen and co-workers [113,116] have shown that the levels of campesterol and sitosterol are proportional to the total and fractional dietary absorption of cholesterol, determined by continuous [^{14}C]cholesterol/[^{3}H]sitosterol feeding. Determination of cholesterol absorption by the latter method is not ideal for large scale studies since it is time consuming and involves the administration of radioactive isotopes. Therefore the measurement of phytosterols may provide a simple and convenient means of measurement of cholesterol absorption.

Recovery of cholesterol from the lipoprotein fractions varied from 71 to 74% as determined by [^{3}H]cholesterol. Linearity was excellent using known amounts of sterol standard added to patient samples, over the range 0.15–20 μmol/l for 7-ketocholesterol, campesterol and sitosterol. The recovery of 0.4 μg of 7-ketocholesterol, campesterol, and sitosterol added to patient sample was 95–99%. The RSD depended on the concentration of sterol and varied from 6.6 to 16% in the concentration range 2–4.5 μmol/l.

It was shown that the ''campestanol'' peak found in serum samples was made up of two sterols: $\Delta^{8,24}$-dimethylsterol precursor of cholesterol synthesis and avenasterol, with traces of sitostanol being found only during sitostanol feeding. Cholestanol and other plant sterol peaks obtained by GLC from sterols in serum appeared to include only the respective sterol.

Vahouny et al. [117] reported the comparative absorption of sitosterol, stigmasterol, and fucosterol and the differential inhibition of cholesterol absorption. The test sterols were extracted by the Folch method from brown algae. After saponification with alcoholic KOH, the non-saponifiable lipids were extracted into hexane. Sterols were precipitated by addition of methanol and recrystallized twice from methanol. (Purity was verified by GC–MS with a packed SE-30 column). The column separated cholesterol, campesterol and stigmasterol but did not resolve effectively sitosterol and fucosterol. These sterols were identified and quantified by an SP-1000 column. Analysis of 24-h lymph collections by GC–MS demonstrated that all three sterols were poorly absorbed to the extent of only 3 to 4% of the administered dose of 50 mg. In contrast, cholesterol absorption under similar conditions was about 42% of the administered dose. Administration of either sitosterol or stigmasterol resulted in an equally effective inhibition of cholesterol absorption (54%). Under identical conditions fucosterol had no effect on absorption of lumenal cholesterol.

Peer et al. [118] have demonstrated that mice lacking the oxysterol receptor, LXRa, lose their ability to respond normally to dietary cholesterol in excess of that which they synthesize de novo.

5.3. Significance

Recently, treatment of familial hypercholesterolemia children with sitostanol pastils has given surprisingly promising results, causing a marked decrease in serum cholesterol level through inhibition of cholesterol absorption [119]. Vanhanen et al. [120], Miettinen and Vanhanen [121], Vanhanen et al. [122] and Gylling and Miettinen [123] have developed a sitostanol ester margarine that includes sitostanol in soluble ester form. The significance of the development is that the margarine can replace a small amount of normal dietary fat, causing 20–15% reduction in serum total and LDL cholesterol level of mildly hypercholesterolemic adult subjects [111]. The effect of the sitostanol margarine on the plasma sterols was determined (assessed) at several levels, including total, free and esterified cholesterol. In addition, squalene and non-cholesterol sterols, including Δ^8-cholestenol, desmosterol, and lathosterol, plant sterols (campesterol and sitosterol) and cholestanol, were determined by GLC [13]. For GLC non-saponifiable lipids of plasma were analyzed, as shown earlier, on a 50-m long SE-30 capillary column. Apparently, no analyses of intact cholesteryl or other steryl esters were made. The resulting decrease of hepatic cholesterol was apparently balanced by enhanced LDL receptor activity and up-regulation of cholesterol synthesis. The relative decrease of the serum campesterol proportion was shown to reduce cholesterol absorption efficiency over 60% in non-insulin-dependent diabetes mellitus patients [123].

An important purpose of these studies was the assessment of model compounds, which will allow a more rational approach for isolation of natural hypocholesterolemic sterol analogues or development of synthetic analogues.

Increased level of plasma cholesterol is generally accepted as a marker for hyperlipoproteinemia and atherosclerosis. The association of high plasma cholesterol concentrations and atherosclerosis is best demonstrated in the inherited lipid disorder, familial hypercholesterolemia, where absent or reduced functional tissue low-density lipoprotein receptors result in extraordinarily high plasma low-density lipoprotein cholesterol levels. As a consequence, these individuals show extensive tendon and tuberous xanthomatosis and develop atherosclerosis rapidly and often die because of myocardial infarctions at young ages. Increased level of plasma cholesterol is generally accepted as a marker for hyperlipoproteinemia and atherosclerosis. The association of high plasma cholesterol concentrations and atherosclerosis is best demonstrated in the inherited lipid disorder, familial hypercholesterolemia, where absent or reduced functional tissue low density lipoprotein receptors result in extraordinarily high plasma low-density lipoprotein cholesterol levels. As a consequence, these individual show extensive tendon and tuberous xanthomatosis and develop atherosclerosis rapidly and often die because of myocardial infarctions at young ages.

Furthermore, plasma accumulation of oxosterols either from enzymic or chemical oxidation has come to be associated with other abnormalities in plasma lipid and lipoprotein metabolism, including inhibition of cholesterol and bile acid biosynthesis, as well as abnormal clearance and accumulation of the low density lipoprotein by macrophages and infiltration of blood vessel walls.

Inhibition of cholesterol biosynthesis is associated with the plasma appearance of the precursor sterols, while inhibition of cholesterol conversion into bile acids is associated with the plasma appearance of the intermediate oxidation products of cholesterol. The increases in the uncommon plasma sterols while highly significant nevertheless may represent only minor components of plasma sterols, which require highly sophisticated methodology for their detection, identification and quantification.

6. Plant sterol metabolism

The plant sterols possess steroid ring structures identical to those of cholesterol and are known to undergo esterification to fatty acid esters presumably via ACAT and LCAT, although specific demonstrations of these transformations are lacking. Likewise, the plant sterols are believed to be subject to reduction of the double bond with the formation of 5α-stanols. However, these transformations take place at rates much slower than those affecting cholesterol.

6.1. Conversion to bile acids

There is evidence that an ethyl group at C_{24} obstructs the conversion of sitosterol to C_{24} bile acids [124–126]. Several previous studies have shown that sitosterol is converted into polar compounds in the bile acid fraction of bile [127,128]. Muri-Boberg et al. [126] and Lund et al. [129] have identified most of the polar products. It was shown that that the polar products resulting from [4-^{14}C]sitosterol were di- and trihydroxylated C_{21} bile acids. Using mass spectrometry, nuclear magnetic resonance (NMR), stereospecific dehydrogenases and reagents, the major trihydroxylated C_{21} bile acids were identified as 5β-pregnan-3α,11β,15β-triol-21-oic acid 5β-pregnan-3α,11β,15α-21-oic acid, and 5β-pregnan-3α,12α-diol-21-oic acid. Considerably less C_{21} bile acids were formed from labeled sitosterol in male than in female Wistar rats. The C_{21} bile acids formed in male rats did not contain a 15-hydroxyl group. Since Lund et al. [130] have shown that such C_{21} bile acids are also formed from labeled cholesterol, the possibility exists that the C_{21} bile acids are formed from both cholesterol and plant sterols. By analysis of purified most polar products of [4-^{14}C]sitosterol by radio-gas chromatography and the same products of 7α,7β-[$^{2}H_2$]sitosterol by combined gas chromatography–mass spectrometry, Muri-Boberg et al. [131] could identify two major metabolites as C_{21} bile acids. One metabolite had three hydroxyl groups (3α, 15, and unknown), and one had two hydroxyl groups (3α, 15) and one keto group. A possible explanation for the formation of C_{21} bile acids from sitosterol in rat liver is the hypothetical mechanism shown in Fig. 18 [131,132].

After intravenous injection of deuterium-labeled sitosterol in bile fistula female Wistar rats, the isolated C_{21} bile acids were found to contain very little isotope. This is in contrast to early studies by Salen et al. [133] which claimed efficient formation of cholic and chenodeoxycholic acid from intravenously administered [22,23-^{3}H]sitosterol. In a reinvestigation with [4-^{14}C]sitosterol, Muri-Boberg et al. [126] could not find any significant conversion into labeled C_{24} bile acids in two healthy human subjects. The results suggested that healthy human subjects, like other mammalian species studied, have little or no capacity to convert sitosterol into the normal C_{24} bile acids. A conversion of plant sterols to C_{24} bile acids could theoretically occur following dealkylation of the sitosterol to cholesterol before conversion to bile acids. Such a dealkylation occurs in some worms and crabs [134,135], but attempts to demonstrate such pathways in mammals had failed.

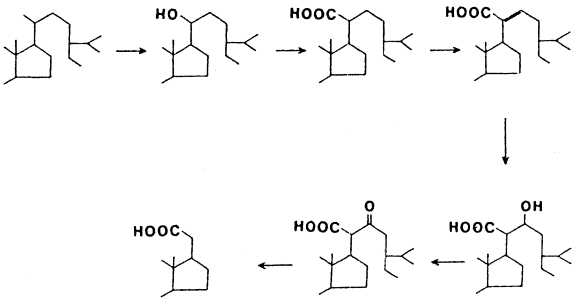

Fig. 18. Structures of C_{21} bile acids produced from cholesterol and from plant sterols. Reproduced from Ref. [131] with permission.

6.2. Conversion to steroid hormones

Muri-Boberg et al. [126] and Lund et al. [130] showed that conversion of labeled sitosterol into C_{21} bile acids also occurred in adrenalectomized and overiectomized rats, indicating that endocrine tissues were not involved. In this connection it may be noted that Subbiah and Kuksis [136] had demonstrated the conversion in rat testes of [4-^{14}C]sitosterol into progesterone, pregnenolone, 17α-progesterone and other polar steroids at rates comparable to those observed with [4-^{14}C]cholesterol. It was concluded that the plant sterol was converted to steroid hormones by a mitochondrial enzyme system of the rat testes which has been shown to cleave the side chain of cholesterol at C_{20} to yield pregnenolone and isocaproic aldehyde. Fig. 19 gives the chemical structures of pregnenolone and progesterone along with those of cholesterol and dihydroxycholesterol, their precursors in the rat testes as reported by Subbiah and Kuksis [136].

6.3. Conversion to 5β-sterols

Under the influence of gut bacteria, the plant sterols along with cholesterol become subject to

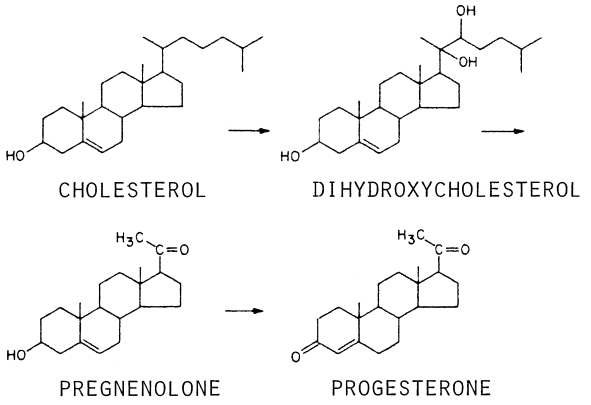

Fig. 19. Biosynthesis of pregnenolone and progesterone from cholesterol.

5β-transformation. Quantitative assessment of cholesterol metabolism necessitates separate determination of the fecal steroids from these two sources. This is readily accomplished by a combined application of TLC and GLC. TLC greatly simplifies the identification and quantification of the sterols in the fecal unsaponifiable matter. Miettinen [9] has used Florisil plates, which were developed with diethyl ether–heptane (55:45, v/v) and sprayed with Rhodamine G. Viewing under UV light revealed three bands (top to bottom): fraction 1, 3-ketocoprostanone and 3-keto derivatives of plant sterols; fraction II, coprostanol and ring saturated 5β-derivatives of plant sterols; fraction III, cholesterol, campesterol, stigmasterol, sitosterol and corresponding ring saturated 5α-sterols. The individual TLC bands were examined by GLC using a 46-m long glass capillary SE-30 column which was temperature programmed from 170 to 265°C at 20°C/min. Fig. 20 shows the GLC resolution of the cuprostanones, coprostanols and cholesterols on a 46 m capillary column. The sterols originating from cholesterol (coprostanol, epicoprostanol, coprostanone, cholesterol and cholestanol) are quantitatively separated from the plant sterols and their bacterial conversion products. Bacterial transformation products of plant sterols are consistently present in large amounts in every fecal sterol sample. On the capillary column, cholesterol and methylcoprostanol (peaks 5 and 7) are clearly separated, allowing quantification even of cholestanol peak (peak 6).

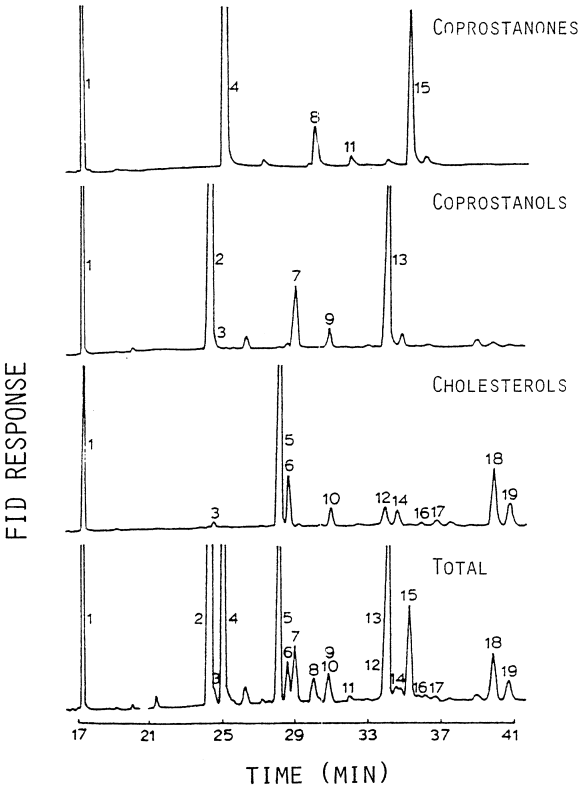

Fig. 20. GLC of fecal neutral sterol TMS derivatives on an SE-30 capillary column following preliminary TLC resolution of the coprostanones, coprostanols and cholesterol fraction from a total fecal sterol mixture. The peak identification is as follows: 1, 5α-cholestane (internal standard); 2, coprostanol; 3, epicoprostanol; 4, coprostanone; 5, cholesterol; 6, cholestanol; 7, methylcoprostanol (formed from campesterol); 8, methylcoprostanone; 9, ethylcoprostenol (formed from stigmasterol); 10, lathosterol; 11, ethylcoprostenone; 12, campesterol; 13, ethylcoprostanol (formed from sitosterol); 14, campestanol (5α); 15, ethylcoprostanone; 16, stigmasterol; 17, stigmastenol (5α); 18, sitosterol; 19, sitostanol (5α). GLC conditions: a glass capillary SE-30 column (46 m×0.3 mm I.D.) was temperature programmed from 170°C to 265°C at 20°C/min. The trimethylsilyl ethers were resolved with helium as carrier gas. Reproduced from Ref. [12] with permission.

6.4. Significance

The significance of the metabolic transformations of the plant sterols in the animal and human tissues remains largely unknown. It is generally believed that the plant sterols interfere with the metabolism of cholesterol. However, certain specific effects of plant sterols have also been recorded. Field et al. [137]

have examined the effect of micellar sitosterol on cholesterol metabolism in CaCo-2 cells and have noted several specific effects, which they have reviewed in the light of previous investigations. However, none of the effects on cholesterol metabolism were attributed to specific sitosterol transformation products. It must therefore be concluded that the metabolic transformation products of sitosterol appear to be of little or no importance to the well being of the animal or to the sitosterolemia patient (see above).

It is also unknown whether or not the oxidation products of plant sterols have any specific metabolic effects. There is evidence that the plant sterols in vitro yield the same oxidation products as cholesterol (see above). What role, if any, would the structure of the side chain play in realizing any metabolic effects of oxidized plant sterols remains to be determined.

7. Conclusions

In reviewing the methods employed for the analysis of the non-cholesterol sterols of blood plasma and diet, it becomes obvious that there is no single method that can be applied to all compounds. Difficulties are experienced at all stages of the analytical process. Thus, a preparation of a plasma lipoprotein by ultracentrifugation is likely to lead to peroxidation of some of the sterol components, and addition of antioxidants may not always help. The selection of organic solvents for the extraction of the sterols must exclude ethers that are readily peroxidized and contribute to the peroxidation of the solutes. Again addition of an antioxidant may not overcome the oxidative stress. The saponification of the esters may cause destruction of the oxosterols unless the reaction is performed at low temperature. Gas chromatography appears to have been the most popular method for the analysis of most types of the sterols, but the high temperatures involved have resulted in the destruction of sensitive samples. While the simpler sterols are stable enough to be analyzed in their free form, the oxosterols require derivatization. Incomplete separation of isomers remains a problem and combinations of gas chromatography with mass spectrometry do not provide a complete answer. HPLC has been rather incompletely explored, nevertheless some remarkable separations have been achieved. It should proved the method of choice for the more polar and temperature sensitive sterols. The best approach appears to be provided by combinations of analytical methods although the small sample sizes and the low abundance of the sterol of interest may limit the choices to gas chromatography or mass spectrometry. In conclusion, there is a need for individual approaches to the analyses of each compound or a small group of compounds. A precise analysis comes down to the use of a stable isotope labeled standard for each unknown as already practiced in mass spectrometry.

Acknowledgements

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